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Pesticide Testing Guidelines, Subdivision M Microbial and Biochemical Pest Control Agents (Part A Microbial)

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GUIDELINES

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PESTICIDE ASSESSMENT GUIDELINES

Subdivision M

Microbial Pest Control Agents and Biochemical Pest Control Agents

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As a guideline for the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA), Subdivision M provides guidance on developing data on microbial (Part A) and biochemical (PB83-153965) (Part B) pest control agents to determine their fate in the environment and to evaluate potential adverse effects on humans and other nontarget organisms. These guidelines provide detailed information relating to the data requirements listed in 40 CFR Part 158.690 and 158.740, including the conditions under which each data requirement is applicable; the standards for acceptable testing, the information that should be included in a test report; guidance on evaluation and reporting of data and examples of acceptable protocols. In addition, scientific publications are cited in the guidelines to provide useful information for designing test protocols.

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FOREWORD

Subdivision M describes protocols which may be used to perform testing on biochemical and microbial pest control agents to support their registration as pesticides under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA). Protocols are provided for determining the fate of these pesticides in the environment and for evaluating their potential adverse effects on humans and other nontarget organisms. Subdivision M is a nonregulatory companion to 40 CFR Part 158, Data Requirements for Registration. This revision of Subdivision M includes changes to the data requirements to be incorporated in the next revision to 40 CFR Part 158.

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This printing is Part A, Microbial Pest Control Agents. Part B, Biochemical Pest Control Agents, will be released separately.

SUBDIVISION M: GUIDELINES FOR TESTING MICROBIAL AND BIOCHEMICAL PEST CONTROL AGENTS

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INTRODUCTION

I. Contents of Subdivision M.

Subdivision M provides testing and informational guidelines for data to be submitted to support registration of Microbial (Part A) and Biochemical (Part B) pest control agents. The following eight section series and topics are covered:

151 Product analysis
152 Toxicology
153 Residue data
154 Nontarget organism hazards
155 Environmental fate and expression
156 Product performance
157 Experimental use permit data
158 Label development

The Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) establishes the Agency's authority over the distribution and use of pesticide products. Before the Agency can register a pesticide, FIFRA requires the Agency to have sufficient data to determine that the pesticide, when used in accordance with widespread and commonly recognized practice, will not cause (or significantly increase the risk of) unreasonable adverse effects to humans or the environment (Section 3(c)(5) and (7) of FIFRA).

Part 158 of Title 40 of the Code of Federal Regulations (CFR), specifies the kinds of data and information that must be submitted to EPA to support the registration of each pesticide. This subdivision provides detailed information relating to the data requirements listed in 40 CFR 158.690 and 158.740, including the conditions under which each data requirement is applicable; the standards for acceptable testing; the information that should be included in a test report; guidance on evaluation and reporting of data; and examples of protocols. In addition, scientific publications are cited in the guidelines to provide useful information for designing test protocols.

II. Scope of Subdivision M.

For regulatory purposes, the Agency has classified biological and biologically derived pesticides into two major categories: the microbial pest control agents i.e., microorganisms, and the biochemical pest control agents e.g., pheromones, hormones, natural insect and plant growth regulators, and enzymes. Biological and biologically derived pesticides are typically naturally occurring, specific to the target species, and, in the case of biochemicals, typically have unique or non-toxic modes of action.

Because of these factors, the biological pesticides are most appropriately characterized for health and environmental safety by testing schemes which take their unique characteristics into account.

A. Microbial pest control agents (MPCAs). Pesticides referred to as microbial pest control agents include, but are not limited to, bacteria, algae, fungi, viruses, and protozoa as defined in 40 CFR 152.20. The guidelines apply to all microbial pest control agents used as pesticides, including both those that are naturally occurring, and those that are strain improved, either by natural selection or by deliberate genetic manipulation.

These guidelines were developed for MPCAs in order to address the special testing needs for these products. Unlike chemical pesticides, MPCAs may survive and reproduce in the environment, and may infect or cause disease in other living organisms. Thus, the basic testing protocols are designed specifically to detect any of these characteristics. Protocols for further testing emphasize exposure or environmental expression in addition to expanded testing of infectivity and pathogenicity.

B. Biochemical pest control agents (BPCAs).

Biochemical pest control agents generally fall into four biologically functional classes: semiochemicals, hormones, natural plant regulators, natural insect growth regulators, and enzymes. EPA has developed distinct data requirements for BPCAs in order to facilitate the registration of this class of pesticides. BPCAs are generally species specific and control their target pest by means such as growth regulation or mating disruption. In contrast, conventional pesticides are generally developed because they are toxic (usually lethal) to a pest and less attention is given to the selectivity of the pesticides for the target species. Furthermore, most BPCAs are applied at very low rates, are highly volatile, or are applied in bait, trap, or "encapsulated" formulations. Thus, the application of most BPCAs results in less exposure to humans and the environment than that from the use of most conventional pesticides. Therefore, the likelihood of adverse effects from BPCAs will be relatively lower than that for most conventional pesticides.

The regulations concerning data requirements for registration (40 CFR 158) indicate at 158.65 that both biochemical and microbial pesticides are generally distinguished from conventional pesticides by their unique mode of action, low use volume, specificity to the target species, or natural occurrence (as described above). The regulation further states that, for biochemicals, the Agency will, when necessary, evaluate products on an individual basis to determine whether they are biochemical or conventional chemical pesticides.

The Agency has evaluated numerous candidate biochemicals on a product by product basis. In each case, one of the major purposes of this evaluation has been to determine whether, on the basis of the above characteristics, the product can be sufficiently distinguished from conventional chemical pesticides to warrant regulation as a biochemical product. Our experience has shown that no single characteristic is sufficient to define a product as a biochemical. Moreover, certain combinations of characteristics have been found to be more important than others and thus, to date, products determined to be biochemicals have always possessed some combination of the above characteristics. Experience has also shown that unique mode of action and natural occurrence are two of the key characteristics supporting the determination that a product is a biochemical.

III. Background of Subdivision M

A. History of guidelines for biological pesticides. The first microbial pesticide (Bacillus popilliae) was registered in 1948. This pesticide was a naturally occurring bacterium. During the late 1960s and early 1970s, interest in microbial pesticides began to increase. As of 1987, there are 14 microbial pesticides used in about 100 separate products registered for use in agriculture, forestry, mosquito control, and homeowner situations.

In 1974, in recognition of the growing interest in, and concern about microbial pesticides, the Agency began to sponsor a variety of workshops, symposia, and panel discussions aimed at identifying the relevant safety concerns for microbial pesticides. As early as 1978, at an EPA symposium titled "Viral Pesticides: Present Knowledge and Potential Effect on Public and Environmental Health", the need for sensitive identification and detection methods for microorganisms as well as quality assurance provisions were clearly identified.

The Office of Pesticide Programs (OPP) issued a Policy Statement on Biorational Pesticides which was published in the FEDERAL REGISTER of May 14, 1979 (44 FR 23994). In it, OPP recognized microbial and biochemical pesticides as distinct from conventional chemical pesticides, and made the commitment to develop appropriate testing guidelines. In 1979, OPP commissioned an American Institute of Biological Sciences' expert panel to develop a "Human Hazard Evaluation Scheme for Biorational Pesticides." The final report of this expert panel formed the basis for the mammalian toxicology unit of the testing guidelines for microbial pesticides.

OPP completed draft testing guidelines for Microbial and Biochemical Pesticides in 1980. After review by the FIFRA Scientific Advisory Panel (SAP) and public comment, these guidelines were finalized in October, 1982, as Subdivision M of the Pesticide Assessment Guidelines and published through the National Technical Information Service (NTIS) in 1983 (EPA-540/9-82-028). The microbial pesticide portion of the Subdivision M guidelines applies to both naturally occurring and genetically modified pesticides. It was also decided at that time that any additional data that would be required for the registration of genetically modified microorganisms would be determined on a case-by-case basis by EPA.

B. Registration requirements. The Data Requirements for Pesticide Registration, 40 CFR Part 158, were published in the FEDERAL REGISTER of October 24, 1984, (49 FR 42856). This regulation contains data requirements for biochemical pesticides at 158.690 and for microbial pesticides at 158.740. These data requirements were previously reviewed by the FIFRA SAP in October, 1983, as part of Subdivision M of the Pesticide Assessment Guidelines.

C. Field testing requirements. The provisions for obtaining Experimental Use Permits (EUP) for testing pesticides is codified in 40 CFR Part 172. This regulation includes a presumption that an EUP will not be necessary for small-scale field testing (non-food uses on not more than ten acres of land or not more than one surface-acre of water, providing that the water not be used for irrigation purposes, drinking water supplies, or body-contact recreational activities). Such a presumption is appropriate for chemical pesticides which have no independent mobility or reproductive capability and, therefore, when applied in small scale field studies generally have very limited potential for causing adverse effects outside the treated area. Similarly, when used in small scale field tests, naturally-occurring microbial pesticides are subject to natural control or dissipation mechanisms. However, genetically altered microbial pesticides may not be subject to natural control or dissipation mechanisms and thus may be capable of spreading beyond the site of application with the potential for causing adverse effects. Therefore, small scale field studies with these types of microbial pesticides could raise many of the same concerns as more extensive use of conventional pesticides, and the presumption that an EUP is not required may not be appropriate for these pesticides.

To address these issues, the Agency developed an interim policy requiring notification under FIFRA prior to small scale field testing of genetically altered and nonindigenous microbial pesticides in order to determine the need for EUPs prior to release of these MPCAs into the environment. This interim policy was published in the FEDERAL REGISTER of October 17, 1984 (49 FR 40659), and also as part of the Proposal for a Coordinated Framework for Regulation of Biotechnology in the FEDERAL REGISTER of December 31, 1984 (49 FR 50856). This policy was revised in accordance with public comments and was re-published in the FEDERAL REGISTER of June 26, 1986, as part of the Coordinated Framework for Regulation of Biotechnology; Announcement of Policy and Notice for Public Comment (51 FR 23302). The Agency is currently in the process of promulgating amendments to 40 CFR Part 172 to implement this policy.

IV. Revisions to Subdivision M.

The Agency has gained considerable experience in the risk assessment of MPCAs and BPCAs since Subdivision M was published in 1983. Accordingly, the need for revising and updating portions of the guidelines, particularly the sections dealing with microorganisms, has become apparent. This revision is intended to better address the needs of both testing laboratories and OPP scientific staff. The Agency recognizes that further revisions will be needed as we learn more about the behavior of microorganisms in the environment. Accordingly, OPP is actively sponsoring research in this area through the EPA Office of Research and Development.

A key editorial feature of these revised guidelines is that the BPCA and MPCA sections have been separated into two distinct parts of the document. The revised version also reflects an extensive updating of testing guidelines for MPCAs.

In addition, the immunotoxicology section for testing BPCAs has been upgraded from the original version. These immunotoxicology guidelines were presented to the FIFRA SAP for review on March 24, 1987, and their comments have been incorporated. No other changes have been made in the BPCA guidelines than the editorial changes necessary in separating them from the MPCA guidelines.

The revised guidelines utilize the tier testing scheme set forth in 1983 with the original publication of Subdivision M. Three of the major section series, Toxicology, Nontarget Organism Hazard, and Environmental Fate and Expression, use the tiered testing scheme (see Appendix) to ensure, to the best extent possible, that only the minimum data sufficient to make scientifically sound regulatory decisions will be required. The Agency expects most of the MPCAs and BPCAs will require testing only in the first Tier. Moverover, the Agency believes that the Tier I test requirements represent a reasonable approach to evaluating risk related to the use of pesticides, and is one in which negative results would allow a high degree of confidence in the safety of the test agents.

V. Relationship to Other Subdivisions of the Guidelines.

This subdivision describes a complete set of requirements for biochemical and microbial pesticides. In addition to tests that are unique to these classes of pesticides, some tests are identical to those used for conventional pesticides. In these cases, references are made to other subdivisions. Each section series in this subdivision corresponds to a subpart of the guidelines for conventional chemical pesticides.

To illustrate:

Series 151 Product Analysis corresponds to Subdivision D - Product Chemistry;

Series 152 Toxicology corresponds to Subdivision F - Hazard Evaluation: Humans and Domestic Animals;

Series 153 Residue Analysis corresponds to Subdivision O - Residue Chemistry;

Series 154 Nontarget Organism Hazard corresponds to a combination of Subdivision E - Hazard Evaluation: Wildlife and Aquatic Organisms, Subdivision J - Hazard Evaluation: Nontarget Plants, and Subdivision L - Hazard Evaluation: Nontarget Insects;

Series 155 Environmental Fate and Expression corresponds to Subdivision N - Environmental Fate;

Series 156 Product Performance corresponds to Subdivision G - Product Performance;

Series 157 Experimental Use Guidelines corresponds to Subdivision I - Experimental Use Permits; and

Series 158 Labeling Development corresponds to Subdivision H - Labeling for Pesticides and Devices.

A. MICROBIAL PEST CONTROL AGENTS

150A GENERAL INFORMATION

150A-1 Overview.

(a) Scope and purpose. This subdivision describes the kinds of data required by 40 CFR Part 158 to support the field testing and registration of naturally occurring or genetically altered microbial pest control agents (MPCAs). Each section in this subdivision specifies the kinds of data required by Part 158, the standards that the studies must meet, and the conditions under which each study is required as specified in Part 158.

(b) Data requirements for registration. MPCA data requirements are listed in 40 CFR 158.740. Testing is required in the areas of Product analysis, Toxicology, Residue analysis on food crops, and Ecological effects and Environmental expression. These guidelines contain revised data requirements as listed in the Appendix to this document.

Product analysis requirements include the necessary data and information to identify the active ingredient and any inert substances that have been added, and to guard against chemical and biological contamination both prior to registration and during production of the MPCA. This information is required for all MPCAs.

Toxicology requirements are set forth in three tiers. Tier I consists of a battery of short term tests designed to evaluate potential for toxicity, infectivity, and pathogenicity. Tier II is designed to evaluate the particular situation when, in the absence of evidence of pathogenicity, either toxicity or infectivity is observed in Tier I. Tier III contains tests that may resolve issues of known or suspected human pathogenicity and tests for particular adverse effects of intracellular parasites of mammalian cells.

Residue data describe the quantity of MPCA or its associated toxins that might appear on food or feed crops. These data are required only if there are significant human health concerns arising from the toxicology testing.

Ecological effects and environmental expression testing also have been placed into tiers. Tier I consists of maximum dose single species hazard testing on nontarget organisms. If adverse effects are observed in Tier I, then the potential exposure to the MPCA is estimated by means of Tier II testing for population dynamics (fate and expression) in the environment. If Tier II tests show that there may be significant exposure to the MPCA, then Tier III studies to determine a dose-response effect or to examine certain chronic effects will be performed to determine if the minimum infective dose is less than the exposure or if there are other considerations that would decrease the observed effects in the environment. Tier IV tests, under simulated or actual environmental conditions, are to be designed on a case-by-case basis to evaluate any specific problem that can not be resolved by lower tier testing.

(c) Data requirements for field testing.

At the date of these Guidelines, the Agency is in the process of promulgating amendments to 40 CFR Part 172 to implement the policy published in the June 26, 1986 FEDERAL REGISTER (51 FR 23302) for submission of notifications prior to conducting small scale field tests (testing involving 10 acres or less of land or 1 acre or less of water) of certain microbial pesticides (see section III C., above). In the interim, anyone considering small scale field tests of microorganisms covered by the policy should consult with the Agency.

Pursuant to Section 5 of FIFRA and 40 CFR 172.2, the Agency provides for issuance of Experimental Use Permits for large scale field testing of pesticides, including all MPCAs, involving terrestrial application of more than 10 acres or direct application to more than 1 acre of water. The Agency encourages applicants for EUPs to consult with OPP scientists to develop an appropriate testing scheme.

The data required prior to field testing of MPCAs may consist of any, or all, of the tests required for registration since these living microorganisms have the potential to multiply to high exposure levels, depending on the their ability to survive, reproduce and compete for dominance in the environment. However, the Agency recognizes the need to limit testing in the course of development of pesticides and intends to make every effort to restrict field testing data requirements to only those necessary to evaluate that particular field test.

Reduced data requirements for EUPs may be justified on a case-by-case basis by consideration of certain exposure factors (e.g., limited capacity for the MPCA to survive at, or disseminate from, the field test site, and containment or mitigation provisions in the test protocols). Human health and ecological effects testing will be limited to the most likely areas of concern as predicted by a careful consideration of the known properties of the MPCA and similar microorganisms. As much information as possible should be submitted to the Agency to allow for analysis of the potential risks of the field tests. Specific information recommendations for this preliminary assessment of both Notifications and Experimental Use Permit Applications are discussed in 157A of these guidelines.

(d) Principles for determining data needed for field tests.

The full battery of tests for registration of MPCAs was designed to give basic hazard and exposure information for a microorganism with totally unknown properties. In actual practice, an MPCA is usually well identified which may facilitate prediction of the properties and behavior of the MPCA. This is particularly true for the areas of human health and plant pathogenicity. Clinical medicine and agricultural science have identified most microorganisms associated with diseases. If the MPCA is taxonomically similar to a clinically or agriculturally significant microorganism, this particular area of concern should be examined closely, possibly, as provided in 40 CFR 158.75, by requiring additional testing beyond that specified in 40 CFR 158.740. Conversely, if the MPCA belongs to a group of microorganisms that have never been found in association with any disease, a case may be made for reducing, or waiving, the testing requirements for this area of concern.

Part 158 of 40 CFR contains provisions for granting waivers for data requirements in response to specific written requests by applicants (40 CFR 158.45). OPP encourages applicants to discuss their preliminary testing plans with OPP scientists. Waivers of some testing requirements may be appropriate for certain MPCAs. This tailoring of the testing battery on a case-by-case basis relies on both an accurate description of the MPCA and the existence of a reliable taxonomy for the class of microorganism to which it belongs. Some microorganisms have been more closely examined than others and have a larger data base from which to draw conclusions. In addition, certain kinds of microorganisms are more amenable to classification than others. In general, human and plant pathogenic bacteria have been best classified due to their health and economic significance. Other microorganisms, particularly protozoa and fungi, might not be as well studied or described, and it may be difficult to reliably predict their properties from a taxonomic description. In this case, it may be more difficult to justify waiving test requirements.

An additional factor in determining the extent of testing that may be necessary for risk assessment is the degree of species specificity shown by the MPCA. This is of primary importance in assessing ecological risk. Most MPCAs are designed to produce adverse effects against a target species. Careful scientific consideration on a case-by-case basis must be given to the selection of nontarget species to be tested (e.g., beneficial insects, environmentally or commercially significant plants, or wildlife) so as to include species that are most likely to be susceptible.

Because of the difficulty in providing definitive exposure predictions from currently available ecological methods, the Agency takes the approach that hazard testing on nontarget organisms should be submitted initially (Tier I tests). If significant adverse effects are identified in Tier I tests, then ecological exposure tests (Tier II) are performed to attempt to quantify levels of the MPCA to which the susceptible nontarget species may be exposed. Although normally requested at a Tier II level, definitive ecological exposure data showing that the MPCA will not survive or persist in the environment would be good support for a request for waiver of some or all of Tier I testing requirements.

150A-2 Definitions.

(a) Terms used in this subpart are defined in FIFRA, in 40 CFR 162.3, and in the following sections of the guidelines;

60-2 of Subdivision D
70-2 of Subdivision E
80-2 of Subdivision F
90-2 of Subdivision G
100-2 of Subdivision H
110-2 of Subdivision I
120-2 of Subdivision J
140-2 of Subdivision L
160-2 of Subdivision N

(b) In addition, for the purposes of this subdivision:

(1) "Animal" means all vertebrate and invertebrate species, including, but not limited to humans and other mammals, birds, fish, and shellfish.

(2) "Aquatic animals" means all vertebrates and invertebrates that inhabit fresh, estuarine, or marine waters for all or part of their life cycles.

(3) "Aquatic use" means the use of a pesticide in a fresh water, estuarine, or marine aquatic system by either direct application or direct discharge of treated water.

(4) "Biological control agent" means a living organism introduced into the environment to control the population or biological activities of another life form considered to be a pest under sec. 2(t) of FIFRA.

(5) "Dose" means a quantity of material, whether living or not, to be applied to an animal at one time.

(6) "Dosing regimen" means a systematic schedule of doses.

(7) "End-use Product (EP)" is an MPCA-containing product that is registered or intended for direct use or application for pest control purposes. In some cases, an EP is identical to the manufacturing-use product (MP) [the technical grade of the active ingredient (TGAI) or formulation intermediate (FI)]. In other cases, an EP is formulated from the MP by addition of inert ingredients such as antioxidants or other stabilizers, suspending agents, carriers, encapsulating

materials, wetting agents, or anticaking compounds. The intentionally-added inerts may influence MPCA storage stability/ viability as well as deposition and persistence at the end-use site. In some cases, an EP is manufactured via an integrated formulation process, i.e., the MP used for formulation is not a separate registered product.

(8) "Environmental expression" means the extent and manner in which a microorganism establishes and maintains its presence in an ecological habitat.

(9) "Estimated environmental concentration" means an estimate of the concentration of a MPCA occurring in or on various media (i.e., soil, water, air) after pesticide application, as determined from the results of environmental fate or expression Tier II testing.

(10) "ID 50" means the amount of material required to produce overt disease symptoms in 50 percent of the test animals.

(11) "Infectivity" is the ability of a microorganism to cross or evade natural host barriers to infection.

(12) "Maximum expected environmental concentration" means the highest concentration of a pesticide occurring at any given time (usually immediately after application) at a site or in a medium (e.g., water, vegetation, or soil) as determined from the pesticide application rate.

(13) "Manufacturing-use Product (MP)" is a preparation containing the MPCA in question that is used to formulate an end-use product (EP; see above). An MP either is the technical grade of the active ingredient (TGAI) or a formulation intermediate (FI). An FI is a product containing the TGAI to which other ingredients deliberately have been added (e.g. stabilizers, dispersants, diluents).

(14) "Maximum hazard testing" means a testing scheme that is designed to maximize any toxic or pathogenic effects of the test substance on the test (non-target) organism.

(15) "Microbial pest control agent" means any of those microorganisms including (but not limited to) bacteria, fungi, viruses, and protozoa as defined in 40 CFR 162 that are used to control pests.

(16) "Morbidity" means the evident state of disease.

(17) "Moribund" means approaching death.

(18) "Mortality" means the state of an animal or plant in which all vital functions have ceased.

(19) "Natural occurrence" means the presence of an organism in its normal habitat where it grows, develops, and reproduces.

(20) "Pathogenicity" is the ability to inflict injury and damage in the host after infection, and depends on host resistance or susceptibility.

(21) "Plant" means any member of the Kingdom Planta.

(22) "Pure Active Form of Each Ingredient (PAI)" is a preparation containing pesticidally functional units of the MPCA in question obtained after the application of the most rigorous purification procedures. Where techniques are used to genetically alter the MPCA, the genetically altered strain is considered as the basis for defining the pure active form of the MPCA. The purest form of the MPCA that can be obtained is a preparation that is free of any other biological forms and free of contaminating growth media or host substrate material. Chemical pesticidal product from genes that have been engineered into a microorganism also may be considered as separate active ingredients.

(23) "Purest infective form" means that preparation of infective virus containing the least amount of extraneous material.

(24) "Technical Grade of the Active Ingredient (TGAI)" is a material containing the microbial pest control agent (MPCA) in question which is produced commercially, or in a manner equivalent to the planned commercial process, and to which no ingredient intentionally has been added except for purposes of MPCA growth or replication, or typical purification. The TGAI is considered to be the purest preparation resulting from a typical production process, and is the preparation intended for distribution and/or formulation into a formulation intermediate (FI) or end-use product (EP). Where techniques are used to genetically alter the MPCA, the genetically altered strain is considered as the basis for defining the technical grade of the MPCA. Each pesticidal product from genes

that have been engineered into a microorganism also is considered as an active ingredient. The technical grade of each pesticidal product from introduced genes is that form which exists along with the technical grade of the MPCA in question after a typical production process.

(25) "Terrestrial wildlife " means non-domestic birds or animals.

(26) "Toxicity" is the injury or damage in a host caused by a poison or toxin where infection by and/or replication or viability of the microorganism are not necessarily required.

(27) "Toxin" means a poisonous substance, generated by a microorganism, plant, or animal, capable of causing injury or damage when it interacts with host cells.

(28) "Typical end-use product" means a pesticide product representative of a major formulation category (e.g., emulsifiable concentrate, granular product, wettable powder) that contains the active ingredient of the registration applicant's product.

(29) "Virulence factors" mean the traits of a microorganism that allow for pathogenicity.

150A-3 General provisions.

(a) Scope. The standards contained in this section apply to all studies in this subdivision unless modified for use in a specific section.

(b) Basic standards for testing.

(1) Test substance for biological and environmental studies. It is advised that appropriate representatives of the EPA be consulted prior to testing in order to determine the form/purity of the MPCA that should be tested to support the registration of each MP and each EP. It is recognized that certain forms of the MPCA may be inappropriate for certain tests. In general, the form (e.g. vegetative cell, spore, cyst, virion) of the microorganism to be tested should be equivalent to the form that is intended for registration. The test microorganism also should be equivalent to that intended for registration with respect to stage of growth, possession of organelles and appendages, and expression of phenotypic traits (including products from genes that have been intentionally introduced into the microorganism). If significant exposure to other forms of the microorganism is expected, or if changes in

form of the microorganism occur, or are expected to occur in target or non-target species, then these forms also may have to be tested. In general, the following principles should be followed:

(i) Tests requiring use of the technical grade of the active ingredient shall be conducted with the manufacturing-use product if the TGAI and MP are identical, or with the technical grade of the active ingredient used to produce the manufacturing-use or end-use formulated pesticide product if not identical.

(ii) The lot of the substance tested should be the same throughout the duration of the study, and the test sample should be stored under conditions that maintain purity and stability. If the stability of the test substance cannot be maintained for the duration of the study or if, for other reasons, it is not possible to use the same lot throughout the test, subsequent lots of the test substance shall be selected to be as nearly identical to the original lot as practical. Chemical or biological assays shall be performed to ensure composition identity and consistency.

(iii) Each lot of the test substance shall be analyzed, to the limits of technical feasibility, and the name and quantities of ingredients, contaminants, and impurities listed. The determination shall include the quantity of unknown material, if any, so that 100% of the test sample is accounted for. The test substance shall be within the limits of purity, if any, certified in accordance with 151A-15 of this subdivision.

(iv) If the test or control substance is to be incorporated into feed or other vehicle, the period during which the test or control substance is stable or viable in such a mixture should be determined prior to the start of the study. No mixture of test or control substance with the feed or vehicle shall be maintained or used during a period exceeding the known stability or viability of the test or control substance in the mixture. Alternatively, determinations of the stability or viability of the test or control substance in random samples of the diet or vehicle mixture shall be made at least monthly during the study to ensure that proper mixing, formulation, and storage procedures are being followed and that the appropriate concentration of the test or control substance is contained in the mixture.

(v) If the test or control substance is incorporated into feed or other vehicle, its homogeneity and concentration in the diet shall be determined prior to the start of the study and each time a new mixture is prepared. Random samples of the mixture shall be analyzed at least monthly to ensure that proper mixing, formulation, and storage procedures are being followed, and that the appropriate concentration of the test or control substance is contained in the mixture.

(vi) In addition to or in lieu of data otherwise required by this subdivision, the Agency may require, after consultation with the applicant, data derived from testing to be conducted with:

(A) an analytically or microbiologically [e.g., purest infective form (PIF) for viruses] pure grade of an active ingredient;

(B) the labile form of infectious material (e.g., non-occluded virus);

(C) an inert ingredient of a pesticide formulation;

(D) a contaminant or impurity;

(E) a metabolite (from animals or plants) or degradation product of an active or inert ingredient;

(F) the end-use formulated product;

(G) any additional substance (including other pesticides recommended for tank-mixing with the test substance) that enhances the virulence or toxicity of the product for which registration is sought; or

(H) any combination of the substances mentioned in paragraphs (b) (1) (vi) (A) through (H) of this section.

(2) Administration or application of test substance and vehicles.

(i) The manner of administration or application of the test and control substance for biological or environmental testing shall be selected so as to maintain accuracy of the dosage or treatment.

(ii) A vehicle other than water or saline, used to dissolve or dilute the test substance or positive control substance shall be chosen to possess the following characteristics if possible:

(A) It does not alter the absorption, distribution, metabolism, or retention of the test substance;

(B) It does not alter the chemical or biological properties of the test substance or enhance, reduce, or alter the pathogenic or toxic characteristics of the test substance;

(C) At the levels used in the study, it does not produce physiological effects and is nontoxic; and

(D) It should be identical to, or closely resemble the vehicle, if any, used in the pesticide product. It should be identical to the vehicle if possible.

(3) Controls for biological and environmental studies. Controls are used in biological or environmental studies required by this subdivision to ensure that observed effects are associated with the test substance exposure. The appropriate control groups shall be identical in every respect to the treated groups except for exposure to the test substance. In studies involving animals or plants, all controls shall, to the extent possible, be from the same source, be of the same age, receive the same care, and receive the same nutrients as the animals or plants receiving the test substance. To prevent bias, a method to randomly assign organisms to treatment and control groups is required and must be referenced in the report.

(i) Untreated (negative) controls. Untreated (negative) control groups are usually required. Untreated controls receive neither the test substance nor any ancillary material (vehicle).

(ii) Controls treated with inactivated MPCAs. In certain circumstances, deleterious effects may be produced in test animals through a mechanism other than active infection (e.g. anaphylaxis). This control group may provide information useful in determining the mechanism of pathogenesis.

(iii) Vehicle control groups. (A) If a vehicle, other than water or saline, is used to administer the test substance, a concurrent vehicle control group may be required. Vehicle control groups receive treatment with the vehicle alone, and the vehicle is usually administered at the highest level that the vehicle is administered in any test group in the study. Consult individual sections of this subdivision for those tests where a vehicle control is required or recommended.

(B) As provided in paragraph (b) (3) (iii) (A) of this section, the vehicle should be selected on the basis of information establishing that it is non-toxic at the levels used in the study, has no independent physiological effects, and does not alter the chemistry, pathogenicity, or toxicity of the test substance. If, however, there are insufficient data on the effects of the vehicle, testing of the vehicle is required.

(iv) Positive controls. Positive controls generally are not required. These serve as internal quality controls, and demonstrate known test organism sensitivity and response to known toxic or infective agents. They are also used to ascertain if a strain or species reacts similarly to another strain or species when exposed to the same known or standard toxicant or infective agent. Consult individual sections of this subdivision for those tests where a positive control is required or recommended.

(iv) Historical controls. Historical control data are required when the Agency desires information on longevity, spontaneous diseases, or other characteristics of a species or strain selected for study, and for certain comparative or statistical purposes. Consult individual sections of this subdivision for those tests where historical control data are required.

(v) Additional controls may be required as dictated by test design.

(c) Special test requirements. In addition to the data required in this subdivision, data derived from other tests may, under unusual circumstances, be required by the Agency in order to make judgments regarding safety to humans, domestic animals, and other nontarget organisms. Such data will be required where special problems are encountered. Test methods will usually be derived from tests already described or cited in other subdivisions of these guidelines. Such data requests may relate to a proposed pattern of use, a toxicological mode of action, or a unique chemical or microbial property. The data requested will be specific to the problem. Examples of test requirements for unusual circumstances include but are not limited to: certain chemical property data from Subdivision D (61-7), and certain toxicity data from Subdivision F.

150A-4 Reporting of data.

Each test report submitted under this subdivision shall satisfy the reporting requirements of this section, unless a specific section elsewhere in this subdivision directs otherwise.

(a) General requirements.

(1) Identification. Each test shall identify:

(i) The name and address of the laboratory or site where the test was performed and

(ii) The party(s) primarily responsible for any written or other matter contained in the report, and the portions of the report for which each party is responsible.

(2) Verification. Each test report shall be:

(i) Signed by each of the senior scientific personnel, including the laboratory director responsible for performing and supervising the testing and preparing, reviewing, and approving the test report, and;

(ii) Certified by the applicant or an authorized agent of the applicant as a complete and unaltered copy of the report provided by the testing laboratory, whether independent or owned, operated, or controlled by the applicant.

(b) Format and content. The test report shall include all information necessary to provide a complete and accurate description and evaluation of the test procedures and results. The test report shall contain at least four parts: a summary and evaluation of the test results, a description of the test procedures, a listing of the data and information required by each applicable section of this subdivision, and a section in which data and findings are discussed. Metric units of measurement must be used although English units may be included where appropriate. The systems may not be mixed (e.g., mg/qt.).

(1) Summary of test results. This section of the test report shall contain a summary of the data and significant findings.

(2) Description of the test procedure. This section of the test report shall contain a full description of the test procedure. If an applicant believes any of the reporting requirements are not applicable, he shall submit an explanatory statement to this effect. A full description of the test procedure shall include but not be limited to:

(i) Deviation from standards. The report shall indicate all ways in which the test procedure fails to meet applicable standards for acceptable testing contained in this subdivision, and shall state the reasons for such deviations.

(ii) Test methods. Specification of test methods, including a full description of the experimental design and procedures, the length of the study, and the dates on which the study began and ended, shall be stated.

(iii) Substance tested. Identification of the test substance shall be provided, including:

(A) If the test substance is microbiological: scientific name and, to the extent possible, serotype and strain or other appropriate designated type, and, to the extent possible, a qualitative and quantitative determination of composition (including names and quantities of known contaminants and impurities, within technically feasible limits). The determination shall also include quantities of unknown materials, if any, to account for 100% of the sample;

(B) Manufacturer and lot number of the test substance, and relevant properties of the substance tested, (i.e., physical state, pH, stability, and purity); and

(C) Identification and composition of any vehicles or other materials (e.g., diluents, suspending agents, emulsifiers, virulence enhancers) used in administering the test substance.

(iv) Animal and plant data. Animal and plant data shall include:

(A) Species and strain used and reasons for selection of species (if the species is other than the species recommended or required by sections of this subdivision).

(B) Source of supply of test organisms;

(C) Disease history of the test animals;

(D) Description of any pre-test conditioning;

(E) Method used to randomly assign animals or plants to test or control groups;

(F) Numbers of animals of each sex in each test or control group; and

(G) Age and condition of animals or plants at beginning of study.

(v) Environmental conditions. A description of the environmental conditions under which the testing was conducted shall be reported. Further details may be provided by specific testing sections elsewhere in this subdivision.

(vi) Treatments or doses. For studies where test substance applications, treatments, or dosings are made, a complete description of such shall be reported. Further details may be provided by specific testing sections elsewhere in this subdivision.

(vii) Treatment for diseases not caused by the test substance. Test animals or plants with a history of disease shall not be used for microbial pesticide testing. The feed must be antibiotic free. For MPCAs where test organisms have been treated with some agent or manipulated by some system to prevent or control infectious diseases not caused by the test substance, a full description of such treatment or manipulation must be reported. Such description should include:

(A) Identification of the test organisms affected and the disease organism involved;

(B) The nature and severity of the disease;

(C) The date of onset and duration of the disease;

(D) The nature of the treatment or manipulation used to control or eliminate the disease, and the dates of such actions; and

(E) The outcome of the treatments in relation to the disease and to the test results.

(viii) Observations. Method, frequency, and duration of observations made during the study shall be reported. Other related specific information to be reported may be provided by specific testing sections elsewhere in this subdivision.

(ix) Availability of raw data, specimens, and samples of the test substances. The location of all raw data, specimens and samples of the test substances which are retained in accordance with 40-5 and 151A-11, and the name and address of the individual who is responsible for the archives and the name and address of the recognized culture collection, shall be reported.

(x) References. References must be provided for the statistical and other methods employed for analyzing the data, and for any published literature used in developing the test protocol, performing the testing, making and interpreting the observations, and compiling and evaluating the results.

(3) Reporting the results and evaluation of specific tests. The test results and any evaluations of test results should be reported in accordance with the requirements of the individual specific testing sections of this subdivision. Such results and evaluations include all data, information, and analysis necessary to support the registration application and its corresponding product label claims, directions, and precautions. The report must be at such detail that a reviewing scientist has sufficient information to reach an independent conclusion from the data.

(4) Discussion section. This section of the test report will contain a full scientific discussion of any and all positive or unexpected negative results and findings. All aberrant data shall be noted and explanations, based on sound scientific principles, given. Any conclusions arrived at by the study author(s) should be included.

(c) Statistical procedures. (1) General. Appropriate statistical methods shall be used to summarize experimental data, to express trends, and to evaluate the significance of differences in data obtained from different test groups. The methods used shall reflect the current state of the art.

(2) Standard deviation and standard error. All data averages or means shall be accompanied by standard deviations, to indicate the amount of variability in the data. In addition, the standard errors of the means should also be calculated, as they are useful in comparing means from different test groups; however, notations of statistically significant differences accompanied by the confidence level or probability should also be used in place of standard error determinations. Other methods of expressing data dispersion may also be used, when appropriate.

Series 151A: PRODUCT ANALYSIS GUIDELINES FOR MICROBIAL PEST CONTROL AGENTS

The product analysis data requirements for microbial pest control agents under 151A-10 through -16 of this subdivision, to some extent, parallel those for conventional chemical pesticides as specified in Subdivision D. However, due to the unique nature, composition, and mode of action of the MPCAs, there are some important differences. For example, protozoa, bacteria, fungi, and viruses should be identified to the extent possible by taxonomic position, serotype, composition, and strain, or by any other appropriate specific means. This information would take the place of chemical name and structural formula information for conventional pesticides. As a result, the guidelines in 151A-10 through -16 generally reference the corresponding section in Subdivision D and indicate those portions of Subdivision D which do not apply.

In addition, the Agency must be reasonably assured that the methods used and the data submitted are capable of demonstrating that the microbial pesticide used in the field is the same as that which was tested for safety.

151A-1 Overview

(a) Scope of product analysis requirements. This section series outlines guidelines for the submittal of data and information on product analysis in support of applications for microbial pest control agents (MPCA's). These guidelines generally parallel those for conventional chemical pesticide products specified in Subdivision D.

151A-2 through 9 [Reserved]

151A-10 Product Identity and Disclosure of Ingredients

(a) Product identity. As required by 40 CFR 158.740, each application for registration of an MPCA shall contain the following information: the product name; the trade name(s) (if different). The company code number(s) may be given.

(b) Confidential Statement of Formula. As required by 40 CFR 158.740, an application for registration of a product shall contain a Confidential Statement of Formula. This statement shall include the nature and quantity of the active ingredient(s) and diluents and the identity and purpose of inert ingredients such as ultraviolet screens, stickers, spreaders, and other such material. The appropriate EPA form shall be used (i.e., Form 8570-4). The name of each ingredient in the product for which 62-2 of Subdivision D requires certified limits to be established shall be listed. A separate Confidential Statement of Formula is required for each alternate formula of a product. See Section 10 of the Act for requirements related to the protection of trade secrets.

(c) Information on ingredients. Information on ingredients is required by 40 CFR 158.740 to support each application for registration. (1) The identification of the MPCA(s) in the product shall (to the extent possible) include the following:

(i) The taxonomic position, serotype, strain, or any other appropriate designation. The precise test procedures and criteria used for identification [i.e., the morphological, biochemical, analytical (physical, chemical), serological, or other identification means] and the results of such tests should be provided. If the MPCA contains plasmids or other extrachromosomal genetic elements involved in pesticidal activity, pathogenicity, toxicity, etc., these must be identified as well as any known phenotypic characters coded by such elements and their stability. Whether or not a wild type or genetically altered strain is considered to be a new active ingredient will be determined on a case by case basis upon comparison with existing related strains;

(ii) The common, alternative, and superseded names;

(iii) The natural occurrence of the organism, its relationship to other species (particularly those that are pathogenic), and its history;

(iv) A description of the morphological types of the MCPA and any unusual morphological, biochemical, or resistance characteristic(s) of the organism if such characteristic(s) are different from the classic description of the organism;

(v) The amount of MPCA present in the product in recognized units of potency, percentage of weight, units of MCPA per unit weight or volume of product, or other appropriate expression of biological activity.

(vi) The biological properties of the active agent with respect to target species, pest host range, life cycle, and mode of action. With respect to the properties of the microbial agent, any known or potential hazard (such as infectivity) to mammals (including humans), the environment, and nontarget species should be discussed.

(vii) If the MPCA in question has been genetically altered, then, in addition to (c)(1)(i-vi), the methods used to genetically alter the microbe should be provided. In the case of genetically altered products, the identity of the inserted or deleted genetic material (source, nature, size, base sequence data and/or restriction endonuclease map), information on the gene control region, descriptions of the phenotypic traits to be gained or lost, and information as to the genetic stability (reversion tendency or rate of exchange/transfer with other organisms) of the genetically altered chromosomal region or extrachromosomal entity are to be discussed. Genetic material adjacent to the intentionally inserted gene(s) which may have been engineered into the recipient are to be fully characterized and the likelihood of expression must be provided.

(2) An application for registration shall contain the following information on each ingredient, other than the MPCA(s), listed in the Confidential Statement of Formula required in paragraph (b) of this section which is known to be present or which might reasonably be identified in the pesticide product.

(i) Percentage composition (by weight) of each ingredient; the number of units per unit volume or weight is needed for microbial impurities; viability data in terms of PFU, CFU, etc. per unit weight or volume of product;

(ii) Whether the ingredient is an active ingredient, an intentionally added ingredient, or an impurity;

(iii) The chemical name from the Chemical Abstracts 1972-1976 Index of Nomenclature, or other well-defined name. If one or more impurities are microbial agents, then the agents are to be classified/identified according to acceptable nomenclatural systems;

(iv) The Chemical Abstracts (CAS) Registry Number;

(v) The product name, the trade name, and the common name;

(vi) The experimental or internal code number;

(vii) For each active ingredient other than the MPCA, the empirical formula, and the molecular weight or the molecular weight range;

(viii) The structural formula (when known); and

(ix) The composition limits for each ingredient for which 62-2 of Subdivision D requires limits to be certified. This information is to be listed on the Confidential Statement of Formula, EPA Form 8570-4 revised 2/85 (or superseding revisions).

151A-11 Manufacturing Process

As required by 40 CFR 158.740, each application for registration of a manufacturing-use product or end-use product shall contain a description of the basic manufacturing process as required in 61-2 of Subdivision D. The starting and intermediate materials should be listed (including EPA Registration

No. and/or source and purity) together with the steps taken to ensure the integrity of these materials, and the steps taken to limit the extraneous contamination, both chemical and biological, in preparations of the unformulated MPCA. This description shall include the procedures used by the manufacturer to establish the identity and purity of the culture from which the unformulated MPCA is produced, the method of manufacture, and techniques used to ensure a uniform or standardized product. The integrity of the product as determined by specific and sensitive chemical, serological or biological tests is to be demonstrated. If the test is not a recognized standard test, a detailed description of the test together with information regarding precision, specificity, interfering substances, accuracy, and sensitivity must be provided.

A sample of registered MPCAs is to be maintained on deposit in a nationally recognized culture collection. A large initial batch could be preserved in small aliquots to serve as seed stock for subsequent batches. This would minimize contamination and variation (chromosomal and extrachromosomal) caused by serial passage. If the MPCA produces a toxin, this may need to be monitored as a form of quality control.

151A-12 Discussion of Formation of Unintentional Ingredients

As required by 40 CFR 158.740, each registration application shall include the following information:

(a) Theoretical discussion. The theoretical discussion concerns the formation of each substance, aside from the control agents and intentionally added, chemically characterized active and inert ingredients, that might reasonably be present in the pesticide product, as outlined in 61-3 of Subdivision D. Examples of such extraneous materials are: allergens, microbial toxins, and other metabolic products; mutant strains; microbial contaminants with particular reference to potentially infective or antagonistic forms; side products from chemical reactions employed in the manufacturing process, fermentation residues from the growth of bacteria or fungi; extraneous host residues from viruses produced in cell cultures, whole animals, or other living forms; residues of contaminants that remain following the purification or extraction process; and impurities in chemicals used in the manufacturing process. The discussion shall include the procedures used to ensure the purity of the unformulated MPCA preparation; if purity (within reasonable limits) cannot be achieved, then the means of controlling contaminant levels to an acceptable limit must be delineated.

(b) Toxic or sensitizing substances. If substances toxic or sensitizing to humans or other nontarget mammalian species are known or suspected to be present at any stage of the manufacturing process, then data must be submitted to show that the substances do not exist in the final product or exist only in quantities too small to pose any hazard.

(c) Human or animal pathogens. Human or other nontarget animal pathogens such as (but not limited to) Shigella, Salmonella, and Vibrio must not be present at hazardous levels in the technical grade of the active ingredient. If the production method can support the growth of human or animal pathogens, each production batch must be tested for their presence. Each application for registration of a manufacturing-use product or end-use product should contain an analysis of all human or animal pathogens that might be present at potentially hazardous levels in the product before formulation. The application should propose methodology for detecting and/or eliminating these from the product. For example, the method prescribed in 40 CFR 180.1011 to monitor each production batch of Bacillus thuringiensis for the pathogen Bacillus anthracis involves subcutaneous injection of at least 1 million viable microorganisms or spores into each of 5 laboratory mice weighing 17 to 23 grams. Such tests shall show no evidence of infection or injury in the test animals when observed for 7 days following injection.

Methods should be proposed for controlling any hazardous contamination detected in the product. Discarding of a contaminated production batch or treating the formulation may be equally acceptable methods for controlling the contaminating microorganisms.

151A-13 Analysis of Samples

A report on the results of preliminary analysis are required by 40 CFR 158.740 to support the registration of each manufacturing-use product and those end-use products produced by an integrated formulation system.

The guidelines of 62-1 of Subdivision D regarding the analysis of samples shall apply, with the exception that a quantitative serological or other appropriate test for the MPCA may be substituted for the chemical analysis or may be used for confirmation.

The Agency shall entertain waiver requests for analysis down to 0.1% (w:w) based upon the nature of the organism, the manufacturing process, and known or potential ability of the MPCA to produce a toxin or other residue of concern to the Agency.

151A-14 [Reserved]

151A-15 Certification of Ingredient Limits

As required by 40 CFR 158.740, each registration must be supported by a certification of ingredient limits. The guidelines of 62-2 and 62-3 of Subdivision D regarding certification of limits and analytical methods to verify certified limits, respectively, shall apply. The limits for microbial agents (MPCAs and contaminants) should be expressed as: (i) MPCA units per unit weight or volume; (ii) international units of potency per unit weight; and (iii) weight percent of product. Items (i) and (ii) may be determined using biological, genetic, biochemical, serological or other appropriate tests.

Note that two or more methods to verify the certified limits of the MPCA(s) and any microbial impurities may be required. Plate counts (colony- or plaque-forming units/unit weight or volume) or infectivity assays may be necessary for quantitation and to allow preliminary identification. Antibiotic resistance bioassays may eliminate some contamination as well as provide additional identification support. In some cases, especially in the case of genetically altered MPCA's, absolute identification can be achieved only through the use of one or more of the various immunological methods (such as enzyme-linked immunosorbent assay) or molecular probe methods (such as the Southern hybridization procedure or restriction endonuclease mapping). Methods must be specific enough to determine the MPCA in the presence of revertants/mutants and contaminants that may have formed or been introduced during the replication/manufacturing process.

151A-16 Physical and Chemical Properties

(a) When required. Data on physical and chemical properties are required to support the registration of each TGAI, manufacturing-use product, and end-use product. See 40 CFR 158.50 and 158.740 to determine whether these data must be submitted.

(b) Substances tested. Table 1 presents the relevant data pertaining to physical and chemical properties for MPCA preparations. Sections 63-1 through -21 of Subdivision D should be consulted regarding the conduct and the specific provisions of the tests.

151A-17 Submittal of Samples

When required by the Agency, as provided in 40 CFR 158.740, the applicant shall submit a sample of the technical grade of the active ingredient, manufacturing-use product, or end-use product. Directions for storage are to be provided including details involving safety and sample integrity. When required by the Agency, the applicant shall submit a sample of any additional substances in the product as listed in 151A-10(c) of this subdivision. The samples should be sent to: U. S. Environmental Protection Agency, Pesticides and Industrial Chemicals Repository, 2 Triangle Drive, Research Triangle Park, NC 27709, or other facility, as designated.

Table 1. SUMMARY OF PHYSICAL AND CHEMICAL DATA FOR MICROBIAL PEST CONTROL AGENTS

	Test Substance		
Property	Technical grade of the active ingredient	Manufacturing-use product	End-use product
Color	Yes	Yes	Yes
Physical state	Yes	Yes	Yes
Odor	Yes	Yes	Yes
Density or specific gravity	Yes	Yes	Yes
pH	Yes	Yes	Yes
Stability[a]	Yes	No	No
Storage Stability	Yes	Yes	Yes
Viscosity	No	Yes (liquids only)	Yes (liquids only)
Miscibility	No	Yes (emulsifiable liquids only)	Yes (emulsifiable liquids only)
Corrosion characteristics	No	Yes (when packaged in metal, plastic or paper containers)	Yes (when packaged in metal, plastic or paper containers)

[a] Refers to the stability of the MPCA to sunlight, water (such as pH 5, 7, and 9), air, normal and elevated temperatures, and metals and their ions.

Series 152A: TOXICOLOGY GUIDELINES FOR MICROBIAL PEST CONTROL AGENTS

152A-1 Overview

(a) Scope of toxicology requirements. This section series sets forth guidelines for testing to determine the potential for detrimental effects to humans and domestic animals caused by microbial pest control agents.

The testing of MPCAs for possible effects on humans and domestic animals is performed in a sequence of three tiers. The general tier sequence and studies involved for MPCAs are outlined in the Appendix to the document.

(b) Purpose. The purpose of this section is to consider the toxicological concerns that the Agency has for MPCA preparations, and to discuss these concerns in relation to the tiered testing scheme for evaluating pathogenic and toxic effects. Major concerns of the Agency for MPCA preparations that relate to Toxicology are the following:

(1) pathogenicity of the MPCA and of microbial contaminants;

(2) infectivity/unusual persistence of the MPCA and of microbial contaminants;

(3) toxicity of the MPCA, of microbial contaminants, and of preparation by-products.

(c) Tier I testing. The overall purpose of the Tier I studies is to provide a toxicological evaluation of a MPCA preparation with respect to the above three endpoints. The Agency recognizes that pathogenicity, infectivity, and toxicity comprise a complex set of microorganism:host interactions which may not be easily resolved as independent endpoints in all studies. However, it is believed that, in most cases, the data from the Tier I battery of tests will provide a fairly clear evaluation of the potential risks of the MPCA due to pathogenicity, infectivity, and toxicity.

The following Tier I studies are considered appropriate for testing of MPCA preparations:

(1) Acute oral toxicity/pathogenicity study;

(2) Acute pulmonary toxicity/pathogenicity study;

(3) Acute intravenous toxicity/pathogenicity study.

The following Tier I studies are considered appropriate primarily to evaluate the toxicity of chemical components of an MPCA preparation (but are performed on the complete MPCA preparation):

(4) Acute dermal toxicity study;

(5) Primary eye irritation study;

(6) Reporting of hypersensitivity incidents.

The final Tier I study is specific to viruses and is designed to evaluate adverse effects of any viral pesticide control agent to mammalian cell lines:

(7) Cell culture tests with viral pest control agents.

Detailed interim protocols for viral testing have been developed by EPA's Office of Research and Development. Although these protocols have not been completely validated, they are available on request from EPA in order to provide additional guidance for applicants and testing laboratories.

(d) Tier II testing. Tier II testing is designed to examine the situation where Tier I testing indicates that the MPCA exhibits infectivity or toxicity without any evidence of pathogenicity.

(1) Infectivity/unusual persistence. Data from acute toxicity/pathogenicity studies may indicate that the MPCA is able to infect test animals without eliciting definite signs of pathogenicity or toxicity, or the MPCA may be observed to persist in test animals for periods longer than would normally be expected. The Agency considers that these observations are sufficient to warrant subchronic testing, Tier II, in order to determine whether repeated exposure to the MPCA preparation is sufficient to cause toxic or pathogenic effects. Subchronic studies also may be required if an MPCA preparation is unable to be sufficiently freed of contaminating microorganisms, or contaminating microorganisms are insufficiently characterized, or there is sufficient reason to believe that microbially-produced toxins exist in the preparation, and these toxins would not exert effects in test animals after acute exposure in the Tier I studies.

The Agency does not choose to set statistical limits to delineate the meaning of "significant infectivity" or to define "unusual persistence" by selecting minimum periods of time for clearance of a microorganism from a test animal. It is believed that these terms are best defined in the context of data that are obtained from the battery of Tier I studies, and in consideration of the type of microorganism and the route of exposure. In general, the MPCA can be considered as "infective" if evidence is obtained that shows the microorganism can cross or evade natural host barriers to infection. Evidence of replication of the microorganism in the host also bears on the interpretation of whether it is infective for the test animal.

The Agency recognizes that certain forms of microorganisms (e.g. spores) may be cleared from the host animal at a rate slower than vegetative forms of the same microorganism. In addition, in the Tier I tests, it is expected that certain MPCAs may be isolated from the test animal, even at the end of the recommended observation periods. Such isolations should not automatically trigger Tier II testing, the data will be interpreted in light of the decline curves generated, and also in light of any evidence that the MPCA replicates in the host animal.

(2) Toxicity. Toxicity of a MPCA preparation to test animals may be caused by substances produced by, or which are constituents of, the MPCA or any contaminating microorganism; or by substances that are

present as non-biological components of the preparation. The Tier I toxicity/infectivity studies are designed to detect acute toxic effects of biological or non-biological components of a MPCA preparation, in the absence of signs of pathogenicity or infectivity. The acute dermal toxicity study is designed primarily to evaluate toxic effects due to chemical components of the MPCA or to non-biological components of a MPCA preparation. The Agency believes that only rarely would it be expected that a microorganism could bypass the skin barrier, or infect epithelial cells, of a healthy host animal solely by virtue of being placed in contact with the skin. An acute intravenous toxicity/pathogenicity study has been placed in Tier I, to evaluate possible adverse effects of a MPCA preparation once the skin barrier is deliberately bypassed.

If toxic effects are observed in the Tier I studies, in the absence of signs of infectivity or pathogenicity, then a Tier II acute toxicity study normally is required, with the toxic components of the MPCA preparation being used as the dosing material. Additional studies designed solely for evaluating toxicity effects of MPCA preparations are not included in the Tier II or Tier III testing scheme for microbial pesticides because appropriate test protocols already exist in Subdivision F guidelines for testing of conventional chemical pesticides, or in the portion of these guidelines for testing of biochemical pest control agents. These guidelines can be consulted, if required, to provide a full evaluation of toxicity due to components of a MPCA preparation.

(e) Tier III testing. In general, if it is determined from Tier I test results that the MPCA is pathogenic for the test animals, then the Agency is to be consulted to determine the next appropriate course of action. It has been the Agency's experience, to date, that recognized mammalian pathogens have not been considered as likely candidates for registration as pesticides. However, it is not inconceivable that recognized pathogens of mammals may be considered for development as MPCAs, for instance, as rodenticides. In such cases, careful consideration and thorough evaluation for pathogenic effects of the MPCA for nontarget mammals is to be provided. Also, the Agency realizes that certain recognized mammalian pathogens that have been rendered non-pathogenic by recombinant DNA or equivalent techniques, may be considered for use as microbial pesticides. In these cases, the effectiveness of the disarming procedure will have to be clearly established.

Viruses and protozoa require special attention for the following reasons:

(i) viruses are obligate intracellular parasites, and protozoa may be obligate or facultative intracellular parasites;

(ii) these intracellular parasites may be less amenable to sufficient taxonomic identification;

(iii) preparations of these MPCAs that are free of contaminating organisms or cellular debris may be difficult to obtain; and,

(iv) they may be present as contaminants in preparations of other MPCAs.

The studies that comprise Tier III primarily are designed for evaluating the potential of MPCAs, or microbial contaminants of MPCA preparations, that are recognized to be intracellular parasites of mammalian cells, or are shown to be, by data resulting from conducting the lower tier studies. It is expected that registrants would not ordinarily pursue registration of such MPCAs. The Tier III studies also may be appropriate for evaluating known mammalian parasites that have been disarmed of pathogenic traits by use of recombinant DNA or equivalent techniques.

The Agency is concerned about proper characterization and identification of microorganisms that are present as contaminants in MPCA preparations, including formulations and newly-produced batches of any MPCA. Appropriate quality control procedures and appropriate biological analyses shall be employed so to ensure that adverse human and mammalian health effects will not occur from use of MPCA products contaminated with other microorganisms. Also, records of biological analyses shall be maintained and made available to the Agency if required.

(4) Other issues.

(i) Identification and characterization of MPCAs. A sufficient and appropriate taxonomic evaluation of the MPCA is required for an initial assessment of potential adverse health effects to mammals, including humans. In addition to the requirements of 151-20 to -27, data on the effects of temperature range on growth of the MPCA also are useful in determining whether the MPCA may survive or grow at mammalian body temperatures.

If the MPCA is genetically altered, then the methods used to derive the product are to be described in detail; and the correct structure of the gene construct in the engineered MPCA product is to be confirmed. The requirements set forth in 151A-10 to-17 for genetically altered MPCAs also must be met.

(ii) Allergy/hypersensitivity. The Agency believes that to require reporting of observed allergic responses of humans to MPCA preparations is sufficient to adequately address health problems due to types of these reactions. Based on the information provided in such reports, the Agency would recommend adequate precautions in handling the material. In some instances, the MPCA will be a recognized common allergen. In these cases, the Agency may require proper precautionary label statements. The Agency strongly recommends that appropriate respiratory tract coverings be worn when there might be accidental exposure to aerosols of MPCA preparations. The purpose of the recommended coverings is to minimize unnecessary stimulation of the respiratory system.

(iii) Eye irritation/injury. Appropriate eye protection is recommended when exposure (including accidental exposure) to aerosols of the MPCA preparation may occur. If it is demonstrated (e.g., by appropriate label precautionary statements) that workers/applicators's eyes will be protected by appropriate coverings (e.g., goggles) then the primary eye irritation study (152A-14) may not be required.

152A-2 through -9 [Reserved]

Group 1: Tier I Testing.

152A-10 Acute oral toxicity/pathogenicity study with microbial pest control agents [(MPCA)s]: Tier I.

(a) When required. Acute oral toxicity/pathogenicity data are required by 40 CFR 158.740 to support the registration of each manufacturing-use product and each end-use product, and the technical grade of each active ingredient.

(b) Purpose. In the assessment and evaluation of the toxic or pathogenic characteristics of a MPCA, determination of acute oral toxicity/pathogenicity usually is an initial step. It provides information on health hazards likely to arise from a single exposure by the oral route. The purpose of the acute oral study is to provide initial information on the toxicity, infectivity, and pathogenicity of a MPCA using a single high dose exposure and an adequate post-exposure observation period.

(c) Definitions.

(1) "Acute oral toxicity" and "acute oral pathogenicity" are the adverse effects occurring from the oral administration of a single dose of a MPCA. Acute oral toxicity also may be the adverse effects occurring from the oral administration of a microbially produced substance, or from other ingredients in any test substance.

(2) "Dose level" is the amount of MPCA administered. It is expressed as units of the microorganism administered per test animal.

(3) "Units of MPCAs". One unit of representative MPCA groups usually would be defined as follows:

(i) vegetative bacterium: a single, viable organism, and usually the entity that produces a single colony forming unit (CFU) on an appropriate semisolid growth medium.

(ii) bacterial or fungal spore, bacterial or protozoan cyst: an intact individual spore or cyst as determined microscopically, and usually the entity that produces a single CFU on appropriate germination medium.

(iii) fungal mycelium: 10^{-9} gram dry weight or, after standardized preparatory procedures, a mycelium-producing entity on semi-solid growth medium.

(iv) virus: an intact, complete virion or a polyhedral body as determined by electron microscopy, and, usually the entity that produces an infective unit (IU) on appropriate host cells or tissues.

(v) protozoa: an intact vegetative organism, spore, or cyst of the members in the various classes of this phylum.

Due to the wide diversity of forms among microorganisms, other definitions of a unit of a MPCA may be equally appropriate.

(d) Principles of the test method. The test MPCA is administered orally by gavage in a single high dose to experimental animals. Subsequent observations of effects and deaths are made and rate of clearance of the MPCA is estimated. Animals that die during the test are necropsied, and at the conclusion of the test the surviving animals are sacrificed and necropsied. Infectivity of the MPCA is evaluated periodically during the test, and at the conclusion of the test.

(e) Substance to be tested.

(1) The technical grade of each active ingredient shall be tested to support the registration of each manufacturing-use product, and each end-use product.

(2) The form (e.g. vegetative cell, spore, cyst, virion) of the MPCA used in testing should be equivalent to the form that is intended for registration or application. To the extent possible, the test MPCA also should be equivalent to the MPCA intended for registration or application with respect to stage of growth, possession of organelles and appendages and expression of phenotypic traits (including products from intentionally introduced genes). If significant exposure to other forms of the MPCA are expected, or if changes in form of the MPCA occur in the host, then these forms also may have to be tested.

(f) Characteristics of the test MPCA. The test MPCA should be thoroughly characterized as required in 151A of these guidelines.

(g) Test procedures.

(1) Animal selection.

(i) Species and strain. Although several mammalian test species may be used, the mouse or the rat are the preferred rodent species. Commonly used laboratory strains should be employed. If another species is used, justification/reasoning for the alternative selection should be provided. All test animals should be free of parasites or pathogens. Females should be nulliparous and non-pregnant.

(ii) Age. Young adult animals should be used. The weight variation of animals used in a test should not exceed ± 20 percent of the mean weight for each sex.

(iii) Sex. Equal numbers of animals of each sex are required.

(iv) Numbers. At least 6 animals (three animals of each sex) should be used. A sufficient number of additional animals should be used to allow for interim sacrifice for infectivity determinations, and also, for control groups.

(2) Control groups.

(i) A concurrent untreated control group of four animals/sex is required. Half of the animals in the control group (i.e., two animals/sex) should be housed separately from the test group of animals dosed with MPCA, and the remainder of the control animals (the "shelf control" group) should be housed with the dosed animals.

(ii) A separate vehicle control group is not required except when the toxicity of the vehicle is unknown.

(iii) Control groups dosed with inactivated MPCA (i.e., rendered incapable of reproduction or germination or excystment) may prove useful to evaluate toxic properties of the MPCA. Inactivation should be done by a means that allows for reasonable maintenance of the structural integrity of the MPCA.

(3) Dosing.

(i) Dose level. One dose level of at least 10^8 units of the MPCA per test animal should be used. If a dose level of at least 10^8 units per test animal is not used, a justification/explanation must be provided.

(ii) Vehicle. The recommended vehicle for the technical grade of each active ingredient is one that allows for maintenance of viability, or germination capability, or excystment capability, or, for intracellular parasites, infection capability in a suitable host.

(iii) Volume. The maximum volume of liquid that can be administered at one time depends on the size of the test animal. In rodents, the volume should not exceed 2 ml/100 g body weight. Variability in test volume should be minimized.

(iv) Dose quantification. Techniques used to quantify the units of MPCA in any dose will depend on the group of microorganisms to which the MPCA belongs. Where possible, determinations of viable, or potentially viable, or infective units in each dose should be made. A measurement of metabolism associated with a defined biomass may be the preferred technique for quantification of mycelial forms of MPCAs. Quantification should be done concurrently with testing.

(4) Exposure.

(i) The test substance should be administered in a single dose by gavage, using a stomach tube or suitable intubation cannula.

(ii) Animals should be fasted overnight prior to test substance administration. After the substance has been administered, food may be withheld for a further 3-4 hours.

(iii) If a single dose is not possible, the dose may be given in smaller portions over a period not exceeding 24 hours. Where a dose is administered in fractions over a period, it may be necessary to provide the animals with food and water, depending on the length of the period.

(5) Observation period. The observation period should be at least for 21 days after dosing. However, the duration of observation should not be fixed rigidly. It should be determined by the type of MPCA administered and its rate of clearance from the test animals. Duration of the observation period also would depend on the time at which signs of toxicity and pathogenicity appear and disappear, and the time to death of the animals.

(6) Observation of animals.

(i) A careful clinical examination of all animals should be made at least once each day.

(ii) Additional observations should be made daily with appropriate actions taken to minimize loss of animals to the study, e.g., necropsy of, and MPCA enumeration from those animals found dead, and isolation of weak or moribund animals.

(iii) Cageside observations should include, but not be limited to, changes in:

(A) the skin and fur;

(B) eyes and mucous membranes;

(C) respiratory system;

(D) circulatory system;

(E) autonomic and central nervous system;

(F) somatomotor activity; and,

(G) behavior pattern.

(H) Particular attention should be directed to observation of tremors, convulsions, diarrhea, lethargy, salivation, sleep and coma.

(iv) Individual weights of animals should be determined shortly before the test material is administered, weekly thereafter, and at death or at interim or final sacrifice. Changes in weight should be calculated and recorded when survival exceeds one day.

(v) The time of death should be recorded as precisely as possible.

(7) Gross pathology. A gross necropsy of all animals should be performed at the time of death or at interim or final sacrifice. All gross pathological changes should be recorded.

(8) Clearance of MPCA. Feces from test animals should be collected soon after dosing and frequently during the study and examined for the presence of the MPCA to estimate clearance of the MPCA after oral administration. Methods (e.g. immunological assays, DNA probes) other than those used for quantification of MPCAs in each dose may prove useful. Recovery values and detection and sensitivity limits should be determined and reported for each technique used.

(9) MPCA enumeration in tissues, organs, and body fluids. Infectivity or persistence should be assessed by using sensitive techniques to determine the presence of the MPCA in tissues, organs, and body fluids. Methods other than those used for quantification of MPCAs in each dose may prove useful. Recovery values and detection and sensitivity limits should be determined and reported for each technique used. Methods selected for MPCA enumeration should, if possible, allow for detection of microbial replication.

(10) Interim sacrifice. For evaluating infectivity and clearance, the MPCA should be enumerated from tissues, organs, and body fluids of three treated animals per sex, sacrificed at three days after, and then at one week intervals after dosing. The number of interim sacrifice periods required will depend on the nature of the test microorganism, and should be sufficient to adequately establish a pattern of clearance. The MPCA also should be enumerated from the tissues, organs, and body fluids of the "shelf control" group at final sacrifice.

(11) Tissues, organs, and body fluids. (i) For infectivity and persistence determinations, the MPCA should be enumerated from the kidney, brain, liver, lung, spleen, blood, representative lymph nodes, and, where appropriate, from lesions and from the injection site. (ii) Other tissues, organs, and body fluids may have to be examined as indicated by the nature of any toxic and pathogenic effects observed.

(i) Data and reporting.

(1) Treatment of results. In addition to the information recommended by 150A-4 of this subdivision and 80-4 of subdivision F, the test report should include the following information:

(i) number of animals at the start of the test;

(ii) time of death of individual animals;

(iii) number of animals displaying other signs of toxicity and pathogenicity;

(iv) description of toxic and pathogenic effects;

(v) definition for one unit of the MPCA used, and the units/test animal in the dosing material;

(vi) body weights and time taken;

(vii) necropsy findings;

(viii) pathology findings, when performed;

(ix) infectivity/persistence findings;

(x) estimate of rate of MPCA clearance;

(xi) description of all enumeration methods used for MPCA detection and quantification; and,

(xii) verification that each enumeration method is sufficiently sensitive to serve as a useful quantitative assay for the MPCA in tissues, organs, and body fluids.

(2) Evaluation of results. An evaluation should include the relationship, if any, between exposure to the test substance and the incidence and severity of all abnormalities, including;

(i) behavioral abnormalities;

(ii) clinical abnormalities;

(iii) gross lesions;

(iv) body weight changes;

(v) mortality;

(vi) toxicity;

(vii) infectivity; and,

(viii) pathogenicity.

(j) Tier progression

(1) If persistent or significant signs of pathogenicity of the MPCA for the test animals is observed in Tier I, acute oral toxicity/pathogenicity tests may be required in non-rodent animal species. Consultation with the Agency to discuss further testing requirements is recommended.

(2) If toxin production by the MPCA is suspected, or if toxin production is indicated by significant or persistent signs of toxicity in the test animals, in the absence of signs of infectivity or pathogenicity, then;

(i) the toxic component(s) of the dosing material is (are) to be identified, and to a practical extent, isolated.

(ii) an acute toxicity study (152A-20) is to be conducted with the toxic component(s).

(3) If significant infectivity or unusual persistence of the MPCA is observed in the absence of signs of toxicity or pathogenicity, then a subchronic (90-day) study (152A-21) is required.

152A-11 Acute dermal toxicity study with microbial pest control agents [(MPCA)s]: Tier I.

(a) When required. Acute dermal toxicity data are required by 40 CFR 158.740 to support the registration of each manufacturing-use product and each end-use product, and the technical grade of each active ingredient.

(b) Purpose. Acute dermal toxicity data provide information on health hazards likely to arise from a single dermal application of soluble or particulate chemicals associated with a preparation of the MPCA, and/or associated with other ingredients in formulations of the MPCA, and/or associated with products from genetic material intentionally introduced into the MPCA.

(c) Definitions.

(1) "Acute dermal toxicity" is the adverse effect occurring during or following a 24-hour dermal exposure to a single dose of a test substance.

(2) "Dose level" is based on the amount of MPCA administered. It is expressed as;

(i) units of the microorganism applied at each test site on the test animal, and as;

(ii) dry weight of applied test substance at a single test site per kilogram body weight of test animal.

(3) "Units of MPCAs". One unit of representative MPCA groups usually would be defined as follows:

(i) vegetative bacterium: a single, viable organism, and usually the entity that produces a single colony forming unit (CFU) on an appropriate semisolid growth medium.

(ii) bacterial or fungal spore, bacterial or protozoan cyst: an intact individual spore or cyst as determined microscopically, and usually the entity that produces a single CFU on appropriate germination medium.

(iii) fungal mycelium: 10^{-9} gram dry weight or, after standardized preparatory procedures, a mycelium-producing entity on semi-solid growth medium.

(iv) virus: an intact, complete virion or a polyhedral body as determined by electron microscopy, and, usually the entity that produces an infective unit (IU) on appropriate host cells or tissues.

(v) protozoa: an intact vegetative organism, spore, or cyst of the members in the various classes of this phylum.

Due to the wide diversity of forms among microorganisms, other definitions of a unit of a MPCA may be equally appropriate.

(d) Principles of the test method. The MPCA in each formulation to be tested is applied in a single high dose to the skin of experimental animals. Subsequently, observations of effects and deaths are made. Animals that die during the test are necropsied, and at the conclusion of the test, the surviving animals are sacrificed and necropsied as indicated by the nature of the toxic effects observed.

(e) Substance to be tested.

(1) The manufacturing-use product shall be tested to support the registration of each manufacturing-use product.

(2) The end-use product shall be tested to support the registration of each end-use product.

(3) The form (e.g. vegetative cell, spore, cyst, virion) of the MPCA used for preparation of the dosing material should be equivalent to the form that is intended for registration or application. To the extent possible, the test MPCA also should be equivalent to the MPCA intended for registration or application with respect to stage of growth, possession of organelles and appendages, and expression of phenotypic traits (including products from intentionally introduced genes). If significant exposure to other forms of the MPCA are expected, then these forms also may have to be tested.

(f) Characteristics of the test MPCA. The test MPCA should be thoroughly characterized as required in section 151A of these guidelines.

(g) Test procedures.

(1) Animal selection.

(i) Species and strain. Although several mammalian test species may be used, the albino rabbit is the preferred species. Commonly used laboratory strains should be employed. If another species is used, the investigator should provide justification/reasoning for the alternative selection. All test animals should be free of parasites or pathogens. Females should be nulliparous and non-pregnant.

(ii) Age. Young adult animals should be used . The weight variation of animals used in a test should not exceed ± 20 percent of the mean weight for each sex.

(iii) Sex. Equal numbers of animals of each sex with healthy intact skin are recommended.

(iv) Numbers. At least 10 animals (five animals of each sex) should be used.

(2) Control groups. (i) Neither a concurrent untreated nor vehicle control group are required except when the toxicity of the vehicle is unknown.

(3) Dosing. (i) Dose level. The test substance should be applied at 2 grams/test animal. If a dose level of less than 2 grams/test animal is used, then a justification/explaination must be provided.

(ii) Vehicle. Where necessary, the formulation to be tested is suspended in a suitable vehicle. An aqueous solution should be used. The recommended vehicle for the end-use product usually is the same material in which the MPCA will be mixed, suspended, or diluted for application.

(iii) Volume. The moisture content of the test material should not be excessive, but should be sufficient to prevent significant drying of the test material during the exposure period, and to insure good contact with the skin.

(iv) MPCA quantification. The numbers of MPCAs in the dose material should be enumerated. Techniques used to quantify the units of MPCA in any dose will depend on the group of microorganisms to which the MPCA belongs. Where possible, determinations of viable, or potentially viable, or infective units in each dose should be made. A measurement of metabolism associated with a defined biomass may be the preferred technique for quantification of mycelial forms of MPCAs. Quantification should be done concurrently with testing.

(4) Exposure duration. The exposure duration should be for approximately 24 hours.

(5) Observation period. The observation period should be at least for 14 days, but should not be fixed rigidly. It should be determined by the toxic reactions, rate of onset and length of recovery period, and thus may be extended when considered necessary. The time at which toxicity signs appear and disappear, and their duration are important.

(6) Preparation of animal skin.

(i) Approximately 24 hours before the test, fur should be removed from the dorsal and ventral area of the trunk of each test animal by clipping or shaving.

(ii) Not less than 10 percent of the body surface area should be cleared for application of the test substance. The weight of the animal should be taken into account when deciding on the area to be cleared and on the dimensions of the covering.

(7) Application of the test substance.

(i) The test substance should be applied uniformly over an area which is approximately 10 percent of the total body surface area.

(ii) The test substance should be held in contact with the skin with porous gauze and a non-irritating tape throughout a 24-hour exposure period. The test site further should be covered in a suitable manner to retain the gauze dressing and test substance and ensure that the animals cannot ingest the test substance. Restrainers may be used to prevent the ingestion of the test substance, but complete immobilization is not recommended.

(iii) At the end of the exposure period, residual test substance should be removed, where practical, using water.

(8) Observation of animals.

(i) A careful clinical examination should be made at least once each day.

(ii) Cageside observations should include, but not be limited to, changes in:

(A) the skin (including signs of irritation) and fur;

(B) eyes and mucous membranes;

(C) respiratory system;

(D) circulatory system;

(E) autonomic and central nervous system;

(F) somatomotor activity; and,

(G) behavior pattern.

(H) Particular attention should be directed to observation of tremors, convulsions, diarrhea, lethargy, salivation, sleep and coma.

(iv) Individual weights of animals should be determined shortly before the test material is administered, weekly thereafter, and at death or at final sacrifice. Changes in weight should be calculated and recorded when survival exceeds one day.

(v) The time of death should be recorded as precisely as possible.

(vi) At the end of the 24 exposure period, and daily thereafter, any signs of skin irritation should be recorded and scored.

(9) Gross pathology. Consideration should be given to performing a gross necropsy of all animals if indicated by the appearance of toxic effects. If done, all gross pathological changes should be recorded.

(i) Data and reporting.

(1) Treatment of results. In addition to the information recommended by 150A-4, the test report should include the following information:

(i) number of animals at the start of the test;

(ii) time of death of individual animals;

(iii) number of animals displaying other signs of toxicity;

(iv) description of toxic effects;

(v) definition for one unit of the MPCA used, and the units in the dosing material;

(vi) dry weight and net weight determinations of the test material applied per kilogram body weight of the test animal;

(vii) body weights and time taken;

(viii) necropsy findings, when performed; and,

(ix) pathology findings, when performed;

(2) Evaluation of results. An evaluation should include the relationsh if any, between the animals exposed to the test substance and the incidence a severity of all abnormalities, including;

(i) behavioral abnormalities;

(ii) clinical abnormalities;

(iii) skin lesions and skin irritation;

(iv) body weight changes;

(v) mortality; and,

(vi) toxicity.

(j) Tier progression.

(1) If evidence of significant and/or persistent toxicity are observed, then the toxic component(s) of the dosing material are to be identified, and to a practical extent, isolated.

(i) An acute toxicity study (152A-20) is to be conducted with the toxic components.

(2) If no toxic effects are observed, then no further testing is required.

152A-12 Acute pulmonary toxicity/pathogenicity study with microbial pest control agents [(MPCA)s]: Tier I.

(a) When required. Acute pulmonary toxicity/pathogenicity data are required by 40 CFR 158.740 to support the registration of each manufacturing-use product and each end-use product, and the technical grade of each active ingredient.

(b) Purpose. Acute pulmonary toxicity/pathogenicity data provide information on health hazards likely to arise from a single exposure by the pulmonary route. The purpose of the acute pulmonary study is to provide initial information on the toxicity, infectivity, and pathogenicity of a MPCA using a single high dose exposure and an adequate post-exposure observation period.

(c) Definitions.

(1) "Acute pulmonary toxicity" and "acute pulmonary pathogenicity" are the adverse effects occurring from intranasal or intratracheal administration of a dose of a MPCA . Acute pulmonary toxicity also may be the adverse effects occurring from intranasal or intratracheal a microbially produced substance.

(2) "Dose level" is the amount of MPCA administered. It is expressed as units of the microorganism administered per test animal.

(3) "Units of MPCAs". One unit of representative MPCA groups usually would be defined as follows:

(i) vegetative bacterium: a single, viable organism, and usually the entity that produces a single colony forming unit (CFU) on an appropriate semisolid growth medium.

(ii) bacterial or fungal spore, bacterial or protozoan cyst: an intact individual spore or cyst as determined microscopically, and usually the entity that produces a single CFU on appropriate germination medium.

(iii) fungal mycelium: 10^{-9} gram dry weight or, after standardized preparatory procedures, a mycelium-producing entity on semi-solid growth medium.

(iv) virus: an intact, complete virion or a polyhedral body as determined by electron microscopy, and, usually the entity that produces an infective unit (IU) on appropriate host cells or tissues.

(v) protozoa: an intact vegetative organism, spore, or cyst of the members in the various classes of this phylum.

Due to the wide diversity of forms among microorganisms, other definitions of a unit of a MPCA may be more appropriate.

(d) Principles of the test method. The test MPCA is administered by intranasal or intratracheal routes in a single high dose to experimental animals. Subsequent observations of effects and deaths are made, and the rate of clearance of the MPCA is estimated. Animals that die during the test are necropsied, and at the conclusion of the test the surviving animals are sacrificed and necropsied. Infectivity of the MPCA is evaluated periodically during the test and at the conclusion of the test.

(e) Substance to be tested.

(1) The technical grade of each active ingredient shall be tested to support the registration of each manufacturing-use product, and each end-use product.

(2) In some formulations, the aerodynamic mean diameter of the particles may be so large as to render pulmonary testing impractical, or unwarranted. In such cases, the test substance may consist only of the purest infective form of the microorganism. However, justification/reasoning must be provided by the investigator to support a request for exempting specific technical grade preparations from testing by intranasal or intratracheal dosing. In other cases, the aerodynamic mean diameter of the particles may be sufficiently large as to warrant intratracheal exposure versus intranasal instillation.

(3) The form (e.g. vegetative cell, spore, cyst, virion) of the MPCA used in testing should be equivalent to the form that is intended for registration or application. To the extent possible, the test MPCA also should be equivalent to the MPCA intended for registration or application with respect to stage of growth, possession of organelles and appendages and expression of phenotypic traits (including products from intentionally introduced genes). If significant exposure to other forms of the MPCA are expected, or if changes in form of the MPCA occur in the host, then these forms also may have to be tested.

(f) Characteristics of the test MPCA. The test MPCA should be thoroughly characterized as required in section 151A of these guidelines.

(g) Test procedures.

(1) Animal selection.

(i) Species and strain. Although several mammalian test species may be used, the mouse or the rat are the preferred rodent species. Commonly used laboratory strains should be employed. If another species is used, the investigator should provide justification/reasoning for the alternative selection. All test animals should be free of parasites or pathogens. Females should be nulliparous and non-pregnant.

(ii) Age. Young adult animals should be used. The weight variation of animals used in a test should not exceed ± 20 percent of the mean weight for each sex.

(iii) Sex. Equal numbers of animals of each sex are required.

(iv) Numbers. At least 10 animals (five animals of each sex) should be used. A sufficient number of additional animals should be used to allow for interim sacrifice for infectivity determinations, and also for control groups.

(2) Control groups.

(i) A concurrent untreated control group is required.

(ii) A separate vehicle control group is not required except when the toxicity of the vehicle is unknown.

(iii) Control groups dosed with inactivated MPCA (i.e., rendered incapable of reproduction or germination or excystment) may prove useful to evaluate toxic properties of the MPCA. Inactivation should be done by a means that allows for reasonable maintenance of the structural integrity of the MPCA.

(3) Dosing.

(i) Dose level. One dose level of at least 10^8 units of the MPCA per test animal should be used. If a dose level of at least 10^8 units per test animal is not used, a justification/explanation must be provided.

(ii) Vehicle. The recommended vehicle for the technical grade of each active ingredient is one that allows for maintenance of viability, or germination capability, or excystment capability, or, for intracellular parasites, infection capability in a suitable host. The recommended vehicle for the manufacturing-use product or end-use product is the same material in which the MPCA will be distributed, mixed, suspended, or diluted for application.

(iii) Volume. The maximum volume of liquid that can be administered via intranasal or intratracheal routes at one time depends on the size of the test animal. In rodents, the volume usually should not exceed 0.3 ml/100 g body weight. Variability in test volume should be minimized.

(iv) Dose quantification. Techniques used to quantify the units of MPCA in any dose will depend on the group of microorganisms to which the MPCA belongs. Where possible, determinations of viable, or potentially viable, or infective units in each dose should be made. A measurement of metabolism associated with a defined biomass may be the preferred technique for quantification of mycelial forms of MPCAs. Quantification should be done concurrently with testing.

(4) Exposure.

(i) The test substance should be administered via an appropriate delivery system in a single dose into the trachea of each test animal, or by intranasal instillation.

(5) Observation period. The observation period should be at least for 21 days after dosing. However, the duration of observation should not be fixed rigidly. It should be determined by the type of MPCA administered and its rate of clearance from the test animals. Duration of the observation period also would depend on the time at which signs of toxicity and pathogenicity appear and disappear, and the time to death of the animals.

(6) Observation of animals.

(i) A careful clinical examination should be made at least once each day.

(ii) Additional observations should be made daily with appropriate actions taken to minimize loss of animals to the study, e.g., necropsy of, and MPCA enumeration from those animals found dead, and isolation of weak or moribund animals.

(iii) Cageside observations should include, but not be limited to, changes in:

(A) the skin and fur;

(B) eyes and mucous membranes;

(C) respiratory system;

(D) circulatory system;

(E) autonomic and central nervous system;

(F) somatomotor activity; and,

(G) behavior pattern.

(H) Particular attention should be directed to observation of tremors, convulsions, diarrhea, lethargy, salivation, sleep and coma.

(iv) Individual weights of animals should be determined shortly before the test material is administered, weekly thereafter, and at death or at interim or final sacrifice. Changes in weight should be calculated and recorded when survival exceeds one day.

(v) The time of death should be recorded as precisely as possible.

(7) Gross pathology. A gross necropsy of all animals should be performed at the time of death or at interim or final sacrifice. All gross pathological changes should be recorded.

(8) Clearance of MPCA. Lungs from test animals sacrificed during the study should be examined for the presence of the MPCA to estimate clearance of the MPCA after dosing. The first analysis should be done on three animals/sex as soon as possible after dosing. Methods (e.g. immunological assays, DNA probes) other than those used for quantification of MPCAs in each dose may prove useful. Recovery values and detection and sensitivity limits should be determined and reported for each technique used.

(9) MPCA enumeration in tissues, organs, and body fluids. Infectivity and clearance should be assessed by using sensitive techniques to determine the presence of the MPCA in tissues, organs and body fluids. Methods other than those used for quantification of MPCAs in each dose may prove useful. Recovery values and detection and sensitivity limits should be determined and reported for each technique used. Methods selected for MPCA enumeration should, if possible, allow for detection of microbial replication.

(10) Interim sacrifice. For evaluating infectivity and clearance, the MPCA should be enumerated from tissues, organs, and body fluids of three treated animals per sex, sacrificed at three days after, and then at one week intervals after dosing. The number of interim sacrifice periods required will depend on the nature of the test microorganism, and should be sufficient to adequately establish a pattern of clearance. The MPCA also should be enumerated from the tissues, organs, and body fluids of the "shelf control" group at final sacrifice.

(11) Tissues, organs and body fluids.

(i) For infectivity and persistence determinations, the MPCA should be enumerated from the kidneys, brain, liver, lungs, spleen, blood, representative lymph nodes, caecum contents, and, where appropriate, from lesions and the inoculation site.

(ii) Other tissues, organs, and body fluids may have to be examined as indicated by the nature of any toxic and pathogenic effects observed.

(i) Data and reporting.

(1) Treatment of results. In addition to the information recommended by 150A-4, the test report should include the following information:

(i) number of animals at the start of the test;

(ii) time of death of individual animals;

(iii) number of animals displaying other signs of toxicity and pathogenicity;

(iv) description of toxic and pathogenic effects;

(v) definition for one unit of the MPCA used, and the units/test animal in the dosing material;

(vi) body weights and time taken;

(vii) necropsy findings;

(viii) pathology findings, when performed;

(ix) infectivity findings;

(x) estimate of rate of MPCA clearance; and,

(xi) description of all enumeration methods used for MPCA detection and quantification.

(xii) verification that each enumeration method is sufficiently sensitive to serve as a useful quantitative assay for the MPCA tissues, organs, and body fluids.

(2) Evaluation of results. An evaluation should include the relationship, if any, between exposure to the test substance and the incidence and severity of all abnormalities, including:

(i) behavioral abnormalities;

(ii) clinical abnormalities;

(iii) gross lesions;

(iv) body weight changes;

(v) mortality;

(vi) toxicity;

(vii) infectivity; and,

(viii) pathogenicity.

(j) Tier progression

(1) If persistent or significant signs of pathogenicity of the MPCA for the test animals is observed in Tier I, acute pulmonary toxicity/pathogenicity studies may be required in non-rodent animal species. Consultation with the Agency is recommended to determine the requirement for any further appropriate testing.

(2) If toxin production by the MPCA is suspected, or if toxin production is indicated by significant or persistent signs of toxicity in the test animals, in the absence of signs of infectivity or pathogenicity, then, (i) the toxic component(s) of the dosing material is (are) to be identified, and to a practical extent, isolated.

(ii) An acute toxicity study (152A-20) is to be conducted with the toxic component(s).

(3) If significant infectivity or unusual persistence of the MPCA is observed in the absence of signs of toxicity or pathogenicity, then a subchronic study (152A-21) is required.

152A-13 Acute intravenous toxicity/pathogenicity study with microbial pest control agents [(MPCA)s]: Tier I.

(a) When required. Acute intravenous toxicity/pathogenicity data are required by 40 CFR 158.740 to support the registration of each manufacturing-use product and each end-use product, and the technical grade of each active ingredient.

(b) Purpose. Acute intravenous toxicity/pathogenicity data provide information on health hazards likely to arise from a single exposure where the skin is bypassed as a barrier. The purpose of the acute intravenous study is to provide initial information on the toxicity, infectivity, and pathogenicity of a MPCA using a single high dose exposure and an adequate post-exposure observation period.

(c) Definitions.

(1) "Acute intravenous toxicity" and "acute intravenous pathogenicity" are the adverse effects occurring from the intravenous administration of a MPCA Acute intravenous toxicity also may be the adverse effects occurring from the intravenous administration of a microbially produced substance.

(2) "Dose level" is the amount of MPCA administered. It is expressed as units of the microorganism administered per test animal.

(3) "Units of MPCAs". One unit of representative MPCA groups usually would be defined as follows:

(i) vegetative bacterium: a single, viable organism, and usually the entity that produces a single colony forming unit (CFU) on an appropriate semisolid growth medium.

(ii) bacterial or fungal spore, bacterial or protozoan cyst: an intact individual spore or cyst as determined microscopically, and usually the entity that produces a single CFU on appropriate germination medium.

(iii) fungal mycelium: 10^{-9} gram dry weight or, after standardized preparatory procedures, a mycelium-producing entity on semi-solid growth medium.

(iv) virus: an intact, complete virion or a polyhedral body as determined by electron microscopy, and, usually the entity that produces an infective unit (IU) on appropriate host cells or tissues.

(v) protozoa: an intact vegetative organism, spore, or cyst of the members in the various classes of this phylum.

Due to the wide diversity of forms among microorganisms, other definitions of a unit of a MPCA may be equally appropriate.

(d) Principles of the test method. The test MPCA is administered by intravenous injection in a single high dose to experimental animals. Subsequent observations of effects and deaths are made, and the rate of clearance of the MPCA is estimated. Animals that die during the test are necropsied, and at the conclusion of the test the surviving animals are sacrificed and necropsied. Infectivity of the MPCA is evaluated periodically during the test and at the conclusion of the test.

(e) Substance to be tested. (1) The technical grade of each active ingredient shall be tested to support the registration of each manufacturing-use product and each end-use product.

(2) The physical nature of some technical grade preparations may render intravenous testing impractical. In such cases, the test substance may consist only of the purest infective form of the microorganism. However, justification/reasoning must be provided by the investigator to support a request for exempting specific technical grade preparations from testing by intravenous dosing.

(3) The form (e.g. vegetative cell, spore, cyst, virion) of the MPCA used in testing should be equivalent to the form that is intended for registration or application. To the extent possible, the test MPCA also should be equivalent to the MPCA intended for registration or application with respect to stage of growth, possession of organelles and appendages and expression of phenotypic traits (including products from intentionally introduced genes). If significant exposure to other forms of the MPCA are expected, or if changes in form of the MPCA occur in the host, then these forms also may have to be tested.

(f) Characteristics of the test MPCA. The test MPCA should be thoroughly characterized as required in section 151A of these guidelines.

(g) Test procedures.

(1) Animal selection.

(i) Species and strain. Although several mammalian test species may be used, the mouse or the rat are the preferred rodent species. Commonly used laboratory strains should be used. If another species is used, justification/reasoning for the alternative selection should be provided. All test animals should be free of parasites or pathogens. Females should be nulliparous and non-pregnant.

(ii) Age. Young adult animals should be used . The weight variation of animals used in a test should not exceed ± 20 percent of the mean weight for each sex.

(iii) Sex. Equal numbers of animals of each sex are required.

(iv) Numbers. At least 6 animals (three animals of each sex) should be used. A sufficient number of additional animals should be used to allow for interim sacrifice for infectivity determinations and also for control groups.

(2) Control groups.

(i) A concurrent untreated control group of two animals/sex is required. A "shelf control group" is not required.

(ii) A separate vehicle control group is not required except when the toxicity of the vehicle is unknown.

(iii) Control groups dosed with inactivated MPCA (i.e. rendered incapable of reproduction or germination or excystment) may prove useful to evaluate toxic properties of the MPCA. If included, inactivation should be done by a means that allows for reasonable maintenance of the structural integrity of the MPCA.

(3) Dosing.

(i) Dose level. One dose level of at least 10^7 units of the MPCA per test animal should be used. If a dose level of at least 10^7 units per test animal, a justification/explanation must be provided.

(ii) Vehicle. The recommended vehicle is one that allows for maintenance of viability, or germination capability, or excystment capability, or, for intracellular parasites, infection capability in a suitable host.

(iii) Volume. The maximum volume of liquid that can be administered by intravenous injection at one time depends on the size of the test animal. Variability in test volume should be minimized.

(iv) Dose quantification. Techniques used to quantify the units of MPCA in any dose will depend on the group of microorganisms to which the MPCA belongs. Where possible, determinations of viable, or potentially viable, or infective units in each dose should be made. A measurement of metabolism associated with a defined biomass may be the preferred technique for quantification of mycelial forms of MPCAs. Quantification should be done concurrently with testing.

(4) Exposure.

(i) The test substance should be administered intravenously via a needle and syringe in a single dose.

(ii) If a single dose is not possible, the dose may be given in smaller fractions over a period not exceeding 24 hours.

(5) Observation period. The observation period should be at least for 21 days after dosing. However, the duration of observation should not be fixed rigidly. It should be determined by the type of MPCA administered and its rate of clearance from the test animals. Duration of the observation period also would depend on the time at which signs of toxicity and pathogenicity appear and disappear, and the time to death of the animals.

(6) Observation of animals.

(i) A careful clinical examination of all animals should be made at least once each day.

(ii) Additional observations should be made daily with appropriate actions taken to minimize loss of animals to the study, e.g., necropsy of, and MPCA enumeration from those animals found dead, and isolation of weak or moribund animals.

(iii) Cageside observations should include, but not be limited to, changes in :

(A) the skin and fur;

(B) eyes and mucous membranes;

(C) respiratory system;

(D) circulatory system;

(E) autonomic and central nervous system;

(F) somatomotor activity; and,

(G) behavior pattern.

(H) Particular attention should be directed to observation of tremors, convulsions, diarrhea, lethargy, salivation, sleep and coma.

(iv) Individual weights of animals should be determined shortly before the test material is administered, weekly thereafter, and at death or at interim or final sacrifice. Changes in weight should be calculated and recorded when survival exceeds one day.

(v) The time of death should be recorded as precisely as possible.

(7) Gross pathology. A gross necropsy of all animals should be performed. All gross pathological changes should be recorded.

(8) Clearance of MPCA. Blood from test animals should be collected during the study and examined for the presence of the MPCA to estimate clearance of the MPCA after dosing. The first analysis should be done on three animals/sex as soon as reasonably possible after dosing. Methods (e.g. immunological assays, DNA

probes) other than those used for quantification of MPCAs in each dose may prove useful. Recovery values and detection and sensitivity limits should be determined and reported for each technique used.

(9) MPCA enumeration in tissues, organs, and body fluids. Infectivity and clearance should be assessed by using sensitive techniques to determine the presence of the MPCA in tissues, organs and body fluids. Methods other than those used for quantification of MPCAs in each dose may prove useful. Recovery values and detection and sensitivity limits should be determined and reported for each technique used. Methods selected for MPCA enumeration should, if possible, allow for detection of microbial replication.

(10) Interim sacrifice. For evaluating infectivity and clearance, the MPCA should be enumerated from tissues, organs, and body fluids of three treated animals per sex, sacrificed at three days after, and then at one week intervals after dosing. The number of interim sacrifice periods required will depend on the nature of the test microorganism, and should be sufficient to adequately establish a pattern of clearance.

(11) Tissues, organs, and body fluids.

(i) For infectivity and persistence determinations, the MPCA should be enumerated from the kidneys, brain, liver, lung, spleen, blood, representative lymph nodes, caecum contents, and, where appropriate, from lesions and the inoculation site.

(ii) Other tissues, organs, and body fluids may have to be examined as indicated by the nature of any toxic and pathogenic effects observed.

(i) Data and reporting.

(1) Treatment of results. In addition to the information recommended by 150A-4, the test report should include the following information:

(i) number of animals at the start of the test;

(ii) time of death of individual animals;

(iii) number of animals displaying other signs of toxicity and pathogenicity;

(iv) description of toxic and pathogenic effects;

(v) definition for one unit of the MPCA used, and the units/test animal in the dose;

(vi) body weights and time taken;

(vii) necropsy findings;

(viii) pathology findings, when performed;

(ix) infectivity findings;

(x) estimate of rate of MPCA clearance;

(xi) description of all enumeration methods used for MPCA detection and quantification, and

(xii) verification that each enumeration method is sufficiently sensitive to serve as a useful quantitative assay for the MPCA in tissues, organs, and body fluids.

(2) Evaluation of results. An evaluation should include the relationship, if any, between exposure to the test substance and the incidence and severity of all abnormalities, including;

(i) behavioral abnormalities;

(ii) clinical abnormalities;

(iii) gross lesions;

(iv) body weight changes;

(v) mortality;

(vi) toxicity;

(vii) infectivity; and

(viii) pathogenicity.

(j) Tier progression

(1) If persistent or significant signs of pathogenicity of the MPCA for the test animals is observed in Tier I, acute intravenous toxicity/pathogenicity tests may be required in non-rodent animal species. Consultation with the Agency is recommended to determine the requirement for any further appropriate testing.

(2) If toxin production by the MPCA is suspected, or if toxin production is indicated by significant or persistent signs of toxicity in the test animals, in the absence of signs of infectivity or pathogenicity, then;

(i) The toxic component(s) of the dosing material are to be identified, and where possible, isolated.

(ii) An acute toxicity study (152A-20) is to be conducted with the toxic component(s).

152A-14 Primary eye irritation study with microbial pest control agents [(MPCA)s]: Tier I.

(a) When required. Primary eye irritation data are required by 40 CFR 158.740 to support the registration of each manufacturing-use product and each end-use product.

(b) Purpose. Primary eye irritation data provide information on possible hazards associated with a single application of the MPCA to the eye, and/or associated with inert ingredients in formulations of the MPCA, and/or associated with products from genes intentionally introduced into the MPCA.

(c) Definitions.

(1) "Eye irritation" is the production of reversible changes in the eye following the application of a test substance to the anterior surface of the eye.

(2) "Eye corrosion" is the production of irreversible changes in the eye following the application of a test substance to the anterior surface of the eye.

(3) "Dose level" is based on the amount of MPCA administered. It is expressed as;

(i) units of the microorganism administered at each test site on the test animal, and, if applicable, as:

(ii) dry weight of applied test substance at each test site per kilogram body weight of test animal.

(4) "Units of MPCAs". One unit of representative MPCA groups usually would be defined as follows:

(i) vegetative bacterium: a single, viable organism, and usually the entity that produces a single colony forming unit (CFU) on an appropriate semisolid growth medium.

(ii) bacterial or fungal spore, bacterial or protozoan cyst: an intact individual spore or cyst as determined microscopically, and usually the entity that produces a single CFU on appropriate germination medium.

(iii) fungal mycelium: 10^{-9} gram dry weight or, after standardized preparatory procedures, a mycelium-producing entity on semi-solid growth medium.

(iv) virus: an intact, complete virion or a polyhedral body as determined by electron microscopy, and, usually the entity that produces an infective unit (IU) on appropriate host cells or tissues.

(v) protozoa: an intact vegetative organism, spore, or cyst of the members in the various classes of this phylum.

Due to the wide diversity of forms among microorganisms, other definitions of a unit of MPCA may be equally appropriate.

(d) Principles of the test method. Each formulation of the MPCA to be tested is applied in a single dose to one eye of each experimental animal, with the untreated eye of each animal serving as a control. The degree of irritation is read and scored at specified intervals and is further described to provide a complete evaluation of the effects.

(e) Substance to be tested.

(1) The manufacturing-use product shall be tested to support the registration of each manufacturing-use product.

(2) The end-use product shall be tested to support the registration of each end-use product.

(3) The form (e.g. vegetative cell, spore, cyst, virion) of the MPCA used for preparation of the dosing material should be equivalent to the form that is intended for registration or application. To the extent possible, the test MPCA also should be equivalent to the MPCA intended for registration or application with respect to stage of growth, possession of organelles and appendages, and expression of phenotypic traits (including products from intentionally introduced genes). If significant exposure to other forms of the MPCA are expected, then these forms also may have to be tested.

(4) If the test formulation contains substances of known corrosive properties, which, for example, have produced definite corrosion or severe irritation in a dermal study, then it need not be tested for eye irritation or infection.

(f) Characteristics of the test MPCA. The test MPCA should be thoroughly characterized as required in section 151A of these guidelines.

(g) Test procedures.

(1) Animal selection.

(i) Species and strain. Although several mammalian test species may be used, the albino rabbit is the preferred species. Commonly used laboratory strains should be used. If another species is used, the tester should provide justification/reasoning for the alternative selection. All test animals should be free of parasites or pathogens. Females should be nulliparous and non-pregnant.

(ii) Number and age of animals. At least six adult animals should be used, unless reasonable justification for using fewer animals is provided.

(2) Control groups.

(i) Separate animals are not needed for an untreated control group, since each test animal serves as its own control.

(ii) A vehicle control group is recommended if the vehicle is known to cause any toxic eye reactions, or if the ocular effects of the vehicle are unknown.

(3) Dosing.

(i) MPCA level. One dose level of the manufacturing-use product containing at least 10^7 units of the MPCA should be applied to each test eye. If a dose level of at least 10^7 units of MPCA is not used, a justification/explanation must be provided. The units of MPCA in the dose for the end-use product are enumerated concurrent with testing.

(ii) Vehicle. The recommended vehicle for the technical grade of the active ingredient is one that allows maintenance of viability, or germination capability, or excystment capability, or, for intracellular parasites, infection capability in a suitable host. The recommended vehicle for the manufacturing-use product or end-use product is the same material in which the MPCA will be distributed, mixed, suspended, or diluted for application.

(iii) Dose level. A volume of 0.1 ml is used where the product to be tested is an MPCA suspension largely comprised of a liquid phase. When the product to be tested is a solid or a slurry, then 0.1 g should be used unless justification for use of other weights of the test substance is provided. The units of MPCA in the dose of each test product must be quantified concurrent with testing.

(iv) Dose quantification. Techniques used to quantify the units of MPCA in any dose will depend on the group of microorganisms to which the MPCA belongs. Where possible, determinations of viable, or potentially viable, or infective units in each dose should be made. A measurement of metabolism associated with a defined biomass may be the preferred technique for quantification of mycelial MPCAs.

(4) Examination of eyes prior to test. Both eyes of each experimental animal provisionally selected for testing should be examined within 24 hours before testing starts. Any animal showing eye irritation or infection, ocular defects, or pre-existing corneal injury should not be used.

(5) Application of the test substance.

(i) The test substance should be placed in the conjunctival sac of one eye of each animal after gently pulling the lower lid away from the eyeball. The lids are then held together for about one second to prevent loss of the material. The contralateral, untreated eye serves as a control. A local anaesthetic to reduce pain may be used prior to instillation of the test substance, provided that evidence can be presented to show that the anaesthetic does not influence the reaction to the test substance. The control eye also should be anaesthetized.

(ii) The eyes of the test animals should not be washed out until 24 hours after instillation of the test substance. At this time, lukewarm water may be used to wash the eyes.

(6) Observation period. The duration of the observation period should not be fixed rigidly, but it should be sufficient to evaluate fully the reversibility or irreversibility of the effects observed. It normally not exceed 21 days after instillation.

(7) Clinical examination and scoring. (i) All eyes should be examined for ocular lesions at 1, 24, 48, and 72 hours and at 4 and 7 days after treatment. If irritation is observed at 7 days, then examinations should be made every 3 days thereafter for 21 days after treatment. If there is no evidence of irritation at 7 days after treatment, the study may be ended. In addition to the observations of the cornea, iris, and conjunctivae, any other observed lesions should be reported. Periocular regions also should be examined for possible lesions. Grading and scoring of ocular lesions are to be done using in accordance with Table 2 (from Draize, et al., 1965).

TABLE 2: GRADES FOR OCULAR LESIONS

CORNEA

(A) Opacity: degree of density (most dense area is taken for reading)

No ulceration or opacity	0
Scattered or diffuse areas of opacity (other than slight dulling of normal luster), details of iris clearly visible	1
Easily discernible translucent area, details of iris slightly obscured	2
Nacrous area, no details of iris visible, size of pupil barely discernible	3
Opaque cornea, iris not discernible through the opacity	4

(B) Area of cornea involved

None	0
One-quarter (or less) but not zero	1
Greater than one-quarter, but less than one-half	2
Greater than one-half, but less than three-quarters	3
Greater than three-quarters	4

Score equals A x B x 5 Total possible maximum = 80

IRIS

(A) Values

Normal	0
Markedly deepened rugae, congestion, swelling, moderate circumcorneal hyperemia, or injection, (any of these or combination thereof), iris still reacting to light (sluggish reaction is positive)	1
No reaction to light, hemorrhage, gross destruction (any or all of these)	2

Score equals A x 5 Total possible maximum = 10

CONJUNCTIVAE

(A) Redness (refers to palpebral and bulbar conjunctivae, cornea, and iris)

Blood vessels normal	0
Some blood vessels definitely hyperemic (injected)	1
Diffuse crimson color, individual vessels not easily discernible	2
Diffuse beefy red	3

(B) Chemosis

No swelling	0
Any swelling above normal (includes nictitating membrane)	1
Obvious swelling with partial eversion of lids	2
Swelling with lids about half closed	3
Swelling with lids from half to completely closed	4

(C) Discharge

No discharge	0
Any amount different from normal (does not include small amounts observed in inner canthus of normal animals)	1
Discharge with moistening of the lids and hairs just adjacent to lids)	2
Discharge with moistening of the lids and hairs, and considerable area around the eye	3

Score equals (A + B + C) x 2 Total possible maximum = 20

Total possible maximum score is the sum of all scores obtained for the cornea, iris, and conjuctivae.

(ii) Examination of reactions can be facilitated by use of a binocular loupe, hand-slit lamp, biomicroscope, or other suitable device. After recording the observations at 24 hours, the eyes of any or all rabbits may be further examined with fluorescein.

(h) Data and reporting.

(1) Data shall be presented in tabular form, showing, for each individual animal:

(i) The irritation scores at the designated observation time;

(ii) A description of the degree and nature of irritation;

(iii) The presence of serious lesions; and

(iv) Any effects other than ocular which were observed.

(2) Evaluation of the results. The eye irritation scores shall be evaluated in conjunction with the nature and reversibility, or otherwise, of the responses observed. The individual scores do not represent an absolute standard for the irritant properties of a material. They shall be viewed as reference values and are only meaningful when supported by a full description and evaluation of the observations.

(3) Test report. In addition to the information required by 150A-4, the test report shall include the following information:

(i) description of the physical nature of the test substance;

(ii) units of MPCA in the dose of each formulation tested, and, where appropriate, the weight of the test substance in each dose;

(iii) species and strain of test animal used;

(iv) narrative description of the degree and nature of irritation or corrosion or lesions or infections observed;

(v) description of any non-ocular effects observed;

(vi) description of the method used to score the irritation (e.g. hand slit-lamp, biomicroscope, fluorescein); and

(i) Tier progression. No further testing is required.

152A-15 Hypersensitivity incidents with microbial pest control agents [(MPCA)s]: Tier I

(a) When required. Data on incidents of hypersensitivity, including immediate-type and delayed-type reactions, of humans or domestic animals that occur during the production or testing of the technical grade of the active ingredient, the manufacturing-use product, or the end-use product should be reported in support of an application for registration. For reporting of incidents taking place after registration, refer to the requirements in connection with sec. 6(a)(2) of FIFRA.

(b) Reporting. The reporting requirements for these incidents should be the same as those for conventional chemical pesticide incident reports as specified in the Pesticide Incident Report form (EPA form number 8550-5, OMB form number 158-R0008). The following information should be provided, if available:

(1) A description of the ingredients (including the MPCA) to which the affected individual(s) were exposed;

(2) The frequency and duration of exposure to the material, and route(s) of exposure;

(3) The time, date, and location of exposure to the material;

(4) The situation or circumstances under which exposure to the material occurred; and,

(5) any clinical observations.

(c) Tier progression. If any incidents of hypersensitivity of humans or domestic animals are observed during the production or testing of the technical grade of the active ingredient, the manufacturinguse product, or the end-use product, then Tier II testing is required. The specific Tier II test requirements will depend on the nature of the observations and will be determined after consultation with the Agency.

152A-15 Cell culture tests with viral pest control agents

(a) When required. Data from cell culture tests with viruses are required by 40 CFR 158.740 to support the registration of each manufacturing-use product and each end-use product. See 40 CFR 158.50 and 158.740 to determine whether these data are to be submitted.

(b) Purpose. Cell culture tests provide information on the ability of viral pest control agents to infect, replicate in, transform, or cause toxicity in, mammalian cell lines.

(c) Definitions

(1) Most infectious form (MIF): The form or preparation of virus that gives optimal infection in the susceptible cell culture or organism. For non-occluded viruses, the MIF is the purified virus or purified tissues obtained from an infected host. For occluded viruses (e.g. baculoviruses, cytoplasmic polyhedrosis viruses, entomopox viruses) the MIF for cell culture or injection into an organism is extracellular virus found in cell culture medium or in infectious hemolymph. The MIF for susceptible insect hosts for infection by natural routes (feeding) is the viral occlusion body.

(2) Viral Toxicity: The ability of a virus to inflict injury or damage to a host cell, where infection by, and/or replication of the virus are not necessarily required. Toxicity can also be the ability of non-viral components of a preparation to inflict injury or damage to a host cell.

(3) Viral Infectivity: The ability of viral genes to become established in a host cell genome, or the ability of viral genes to be expressed in a host cell (resulting in the production of virus-encoded nucleic acids).

(4) Transformation: The detectable modification of a host cell phenotype induced by the presence of viral nucleic acid. Transformed cells are considered to be infected by the virus.

(5) Cytopathic effects (CPE): Any host cell damage or injury resulting from infection of the cell by a virus. These effects can be morphological or biochemical, and include but are not limited to, cell growth, attachment, morphology, nucleus size and shape, and cellular processes such as macromolecular synthesis.

(d) Test standards.

(1) Substance to be tested. The purest, most infectious form (MIF) of the virus should be used. Preparations of insect viruses should be free of insect hemolymph, unless it has been determined that the hemolymph is not toxic to the cell cultures used. The inoculum should be titered by the most sensitive assay available, and in the most permissive host system (cell culture or, if not available, host organism). For testing in the model systems, a minimum of five plaque-forming units (PFU) per cell is required when a plaque assay for the virus is available, or seven times the LD_{50} units when a plaque assay for the virus is not available. If fewer units per cell or organism are used, then justification/reasoning must be provided for using the lesser amount.

(2) Cell cultures. The following cells are recommended: one human line (e.g., WI38); one primary cell type (e.g., foreskin); one primate continuous line (e.g., monkey CV-1 or BSC-1); primary Syrian hamster embryo (SHE) cells (to provide data for the cell transformation assay, described below). One other cell line is to be selected to evaluate potential concerns intrinsic to the specific viral pest control agent, and its intended use. Justification/reasoning must be provided for the selection of this latter cell line.

(3) Toxicity evaluation. Efficiency-of-plating tests should be performed with each cell line. For each cell line, approximately two hundred cells are plated on each of 30 dishes. At 24 hours post-plating, 10 dishes/cell line are exposed to approximately 10^6 units of the virus. Appropriate vertebrate cell culture medium is added to 10 different dishes, and, if applicable, 10 dishes/cell line are exposed to invertebrate medium only. At 1 hour post-exposure, all cultures are fed with the appropriate vertebrate cell culture medium, and then are incubated until control cultures have colonies consisting of at least 25 cells/colony. All cultures then are fixed and stained, and colonies are enumerated.

(4) Infectivity evaluation.

(i) Subconfluent cultures (containing approximately $2x10^5$ cells on 25 cm^2 dishes) of each cell line are exposed to $\geq 1x10^6$ units of the viral pest control agent. Appropriate controls include subconfluent cultures that receive no treatment, and those that are exposed to virus-free inoculation medium. At 7 days and at 14 days post-inoculation, cells are to be subcultured.

(ii) Cell cultures are observed daily for 21 days post-inoculation for appearance of CPE.

(iii) Cultures are quantitatively assayed for the virus on days 1, 2, 5, 7, 14, and 21 post-inoculation.

(A) Cells (entire culture, or $\geq 2x10^5$ cells) are to be assayed in triplicate for viral antigen and nucleic acid.

(B) Cell culture fluid from replicate cultures is to be assayed for infectious virus, using an appropriate susceptible host model system.

(iv) Assays for fate of input virus and for presence of viral protein(s) and nucleic acid.

(A) The enzyme linked immunosorbent assay (ELISA), dot-immunobinding assay (Tijssen, 1985; Hawkes et al., 1982; Kam et al., 1982), protein blot immunoassay (Western transfer) (Smith and Summers, 1981; Volkman and Goldsmith, 1982) or similar assays are recommended for protein determination.

(B) The dot hybridization procedure (Bishop, 1983; Kafatos, et al., 1979; Brandsma and Miller, 1980), Southern hybridization procedure (Southern, 1975; Smith and Summers, 1983), or other similar assay using probes of high specific activity are recommended for nucleic acid determinations.

(v) Controls.

(A) For each cell culture, cells inoculated with a preparation of the inactivated test virus should be analyzed as described for the active test virus.

(B) For each series of tests, the inoculum should be tested in the permissive cell line or host organism as a positive control and for direct reference to the data obtained from the vertebrate cell lines.

(5) Cell transformation assay.

(i) The ability of the viral pest control agent to transform primary Syrian hamster embryo (SHE) cells is to be determined, using an appropriate assay system. If other test systems are used, then justification/rationale must be presented to show that the alternate systems are appropriate.

(ii) Controls.

(A) Transformation of SHE cells with Simian adenovirus 7 (SAV 7) serves as the positive control.

(B) SHE cells treated with cell culture medium alone, and SHE cells treated with a killed preparation of the inactivated viral pest control agent serve as appropriate negative controls. The inactivation procedure must be demonstrated as effective in preventing transformation.

(C) An efficiency of plating test with SHE cells (see above) is considered an appropriate toxicity control.

(iii) If the data show that the test virus modifies the cell phenotype, then cells from cultures derived from morphologically transformed colonies are to be inoculated into hamsters, and tumorigenesis in the host animal is to be evaluated.

(iv) This assay may not be required if, in the infectivity evaluations (d (4), above), it is conclusively demonstrated that viral nucleic acid is not persistent in any of the test cell lines employed.

(c) Data reporting and evaluation. The following information should be provided for each test:

(1) CPE in the cell monolayers.

(i) The appearance of CPE should be described in such a way that virus-induced cell destruction is differentiated from non-specific effects.

(ii) Cultures should be inspected with the aid of a microscope to provide evidence of CPE that should be recorded as:

1+ = suggestive of virus-induced morphologic changes
2+ = definitive morphologic changes
3+ = more than 50% cell degeneration; or
4+ = complete cell destruction

(iii) The $TCID_{50}$ value calculated by an appropriate statistical method. For computation of the infectivity results, only cultures showing a ≥2+ CPE are considered to be infected.

(2) Toxicity evaluation.

(i) Details of all procedures used, including appropriate reagents and materials, and assay sensitivities and limitations.

(ii) Efficiency of plating data of cultures receiving virus, and cultures receiving vertebrate media (control cultures) and invertebrate media.

(iii) Assessment of mitotic process prevention or of interference with chromosomal replication, as indicated for example, by significant reductions in efficiency of plating.

(3) Assay of culture fluid.

(i) Details of procedures used, including a discussion of all data that indicate viral replication.

(4) Data from assays of input viruses.

(i) Details of procedures used for detection of viral antigens and nucleic acids and their persistence in culture, including appropriate reagents and materials, and assay sensitivities and limitations.

(ii) Intracellular concentration of viral antigens and viral nucleic acids, reported as a function of cell number (e.g., viral genome number/cell).

(5) Cell transformation assay.

(i) Details of the protocols used for the cell transformation assay, and reference to tha assay used, if published.

(ii) Control value data, including efficiency of plating results.

(iii) Tumorogenesis data in test animals, if this study is required.

(6) General information to be provided for all tests:

(i) The source of each cell line used;

(ii) Evidence for lack of adventitious agents in cell lines;

(iii) Information on genetic stability of continuous cell lines, and on donors of primary cells.

(d) Tier progression. The requirement for further studies as indicated by the data from these studies also will be based on data from the other test requirements in this Tier.

(1) If the data show that the viral pest control agent is not cytotoxic, nor does it infect, replicate in, or transform any cell culture, then no further testing is required.

(2) If the data show that the viral pest control agent preparation is toxic to any of the test cell cultures, but does not infect, replicate in, or transform any of the cell cultures, then: (i) the toxic component(s) of the preparation may have to be identified; and,

(ii) an acute toxicity study (152A-20) may be required with the toxic component(s).

(3) If the viral pest control agent infects any of the test cell cultures, then reproductive and fertility effects (152A-30), oncogenicity (152A-31), immunodeficiency (152A-32), and primate infectivity/pathogenicity (152A-33) studies may be required.

(e) References.

(1) Bishop, D. H. L. (1983) The application of RNA finger printing and sequencing to viral diagnosis. Curr. Topics Microbiol. Immunol. 104:259-271.

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(3) Casto, B.C. (1968) Adenovirus transformation of hamster embryo cells. J. Virol. 2:376-383.

(4) Hames, B.D. and S.J. Higgens (eds) (1985) Nucleic acid hybridisation. A practical approach. IRL Press, Washington, D.C. (ISBN 0-947946-23-3).

(5) Heidelberberger, et al. (1983) Cell transformation by chemical agents -a review and analysis of the literature. A report of the U.S. Environmental Protection Agency Gene-Tox Program. Mutation Res. 114:283-385.

(6) Kafatos, F.C., C.W. Jones, and A. Efstratiadis (1979) Determination of nucleic acid sequence homologies and relative concentrations by a dot hybridization procedure. Nucleic Acids Res. 7:1541-1552.

(7) Smith, G. and M.D. Summers (1981) Application of a novel radioimmunoassay to identify baculovirus structural proteins that share interspecies antigenic determinants. J. Virology 39:125-137.

(8) Southern, E. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503.

(9) Tijssen, P. (1985) Laboratory techniques in biochemistry and molecular biology. Practice and theory of enzyme immunoassays. Elsevier Science Publishing (ISBN 0-444-80633-4)

152A-17 [Reserved]

152A-18 [Reserved]

152A-19 [Reserved]

Group 2: Tier II Testing.

152A-20 Acute toxicity study with microbial pest control agents [(MPCA)s]: Tier II

(a) When required. Acute toxicity data are required by 40 CFR 158.740 to support the registration of each manufacturing-use product and each end-use project and the technical grade of each active ingredient when significant or persistent signs of toxicity are observed in test animals from Tier I studies, in the absence of signs of significant infectivity or pathogenicity.

(b) Purpose. Acute toxicity data provide information on health hazards likely to arise from a single exposure to toxins or toxic components derived from, or associated with the test substance. The toxic components are to be isolated and identified. The purpose of an acute toxicity study is to determine the median lethal dose, its statistical limits, and slope using a single exposure and a 14-day post-exposure observation period.

(c) Definitions.

(1) "Acute toxicity" is the adverse effects occurring from administration of a single dose of a component, or components, of the test substance.

(2) "Median lethal dose" is a statistically derived single dose of a substance that can be expected to cause death in 50 percent of animals. It is expressed in terms of weight of test substance per unit weight of test animal, and also in terms of the proportion, by weight, of the toxic component(s) in the test substance per unit weight of test animal.

(d) Principle of the test method. The test substance, including toxic components, is administered in graduated doses to several groups of experimental animals, one dose being used per group. Subsequent observations of effects and deaths are made. Animals which die during the test are necropsied, and at the conclusion of the test, the surviving animals are sacrificed and necropsied as indicated by the nature of the toxic effects observed.

(e) Substance to be tested. The toxic component(s) of the MPCA preparation are to be isolated and identified. The test substance will comprise an appropriately purified preparation of the toxic components. The proportion by weight of the toxic components in the test substance is to be determined and reported.

(f) Test procedures. (1) Animal selection. The species and strains of test animal to be used are those in which toxic effects were observed in the acute toxicity/ pathogenicity studies from Tier I.

(2) Route of exposure. The route or routes of exposure should correspond to each and all routes (i.e., oral, dermal, and pulmonary) where toxicity was observed in the acute toxicity/pathogenicity studies from Tier I. A separate acute toxicity test is required for each route of exposure.

(3) Other test standards. (i) The applicable test standards set forth in 152A-10, 152A-11, and 152A-12, and of this Subdivision, and in the corresponding tests in Subdivision F (i.e., 81-1, 81-2, and 81-3) should be followed.

(ii) If acute pulmonary toxicity tests are required, then the applicable standards set forth in 152A-12 of this Subdivision usually will take precedence over the standards set forth in 81-3 of Subdivision F.

(g) Tier progression. The Agency will make recommendations on appropriate further test requirements. In general, further test requirements will be comprised of appropriate tests as described for biochemical pest control agents in this Subdivision or for chemical pesticides as described in Subdivision F.

152A-21. Subchronic toxicity/pathogenicity studies with microbial pest control agents [(MPCA)s]: Tier II.

(a) When required. Subchronic toxicity/pathogenicity studies are required by 40 CFR 158.740 to support the registration of each manufacturing-use product and each end-use product and the technical grade of each active ingredient when significant infectivity and/or unusual persistence of the MPCA is observed in test animals from the Tier I studies in the absence of significant pathogenicity or toxicity. These studies also may be required to provide an evaluation of adverse effects due to microbial contaminants or toxic by-products in a MPCA preparation.

(b) Purpose. Subchronic toxicity/pathogenicity data provide information on health hazards likely to arise from subchronic (90-day) exposure to a MPCA preparation.

(c) Principle of the test method. The test substance is administered daily to experimental animals at a single high dose level for a time period of at least 90 consecutive days. During this period, animals are observed daily to detect signs of toxicity and pathogenicity. Animals which die during the test period are necropsied, and the MPCA is enumerated from appropriate tissues, organs, and body fluids. At the conclusion of the test, surviving animals are sacrificed and necropsied, and appropriate tissues, organs, and body fluids are analyzed for the quantitative presence of the MPCA.

(d) Substance to be tested.

(1) The manufacturing-use product and, if different, the technical grade of each active ingredient shall be tested to support the registration of a manufacturing-use product.

(2) The end-use product shall be tested to support the registration of an end-use product.

(3) Usually, the form of the MPCA to be tested will be equivalent to the form used in the acute toxicity/pathogenicity Tier I studies of this Subdivision, and which resulted in significant signs of infectivity or unusual persistence without accompanying signs of pathogenicity or toxicity.

(e) Test procedures.

(1) Animal selection.

(i) Species and strain. The species and strains of test animal to be used are those in which infectivity/unusual persistence of the MPCA was observed in the acute toxicity/pathogenicity Tier I studies, and in which no significant signs of pathogenicity or toxicity were observed. All test animals should be free of parasites or pathogens. Females should be nulliparous and non-pregnant.

(ii) Age. Young adult animals should be used. The weight variation of animals used should not exceed ± 20 percent of the mean weight for each sex.

(iii) Sex and numbers. At least twenty animals (ten animals of each sex) should be treated with the MPCA.

(2) Control groups.

(i) A concurrent untreated control group is required.

(ii) A separate vehicle control group is not required except when the toxicity of the vehicle is unknown.

(iii) A control group dosed with inactivated MPCA (i.e., rendered incapable of reproduction or germination or excystment) may prove useful to evaluate toxic properties of the MPCA. Inactivation should be done by a means that allows for reasonable maintenance of the structural integrity of the MPCA.

(3) Dosing.

(i) Dose level. A dose level of $\geq 10^8$ units of viable MPCA per test animal is to be administered daily to each test animal. If a dose level of at least 10^8 units of MPCA per test animal is not used, a justification/explanation must be provided.

(ii) Vehicle. The recommended vehicle for the technical grade for each active ingredient is one that allows for maintenance of viability, or germination capability, or excystment capability, or, for intracellular parasites, infection capability in a suitable host. The recommended vehicle for the manufacturing-use product or end-use product is the same material in which the MPCA will be distributed, mixed, suspended, or diluted for application.

(iii) Route of exposure. The oral route is to be used if significant infectivity/ unusual persistence of the MPCA was observed in test animals in the acute oral toxicity/pathogenicity Tier I study (152A-10). The inhalation route (usually intranasal instillation) is to be used if significant infectivity/unusual persistence of the MPCA was observed in test animals in the acute pulmonary toxicity/pathogenicity Tier I study (152A-12).

(4) Observation of animals.

(i) A careful cageside examination of each test animal should be made at least once per day.

(ii) Additional observations should be made daily with appropriate actions taken to minimize loss of animals to the study, e.g., necropsy of, and MPCA enumeration from those animals found dead, and isolation of weak or moribund animals.

(iii) Cageside observations should include, but not be limited to, changes in:

(A) the skin and fur;

(B) eyes and mucous membranes;

(C) respiratory system;

(D) circulatory system;

(E) autonomic and central nervous system;

(F) somatomotor activity; and,

(G) behavior pattern.

(H) Particular attention should be directed to observation of tremors, convulsions, diarrhea, lethargy, salivation, sleep, and coma.

(iv) Individual weights of animals should be determined shortly before the test material is administered, weekly thereafter, and at death or at sacrifice.

(v) Food and water consumption should be determined weekly during the study.

(vi) The time of death should be recorded as precisely as possible.

(5) Gross pathology. A gross necropsy of all animals should be performed at the time of death or at sacrifice. All gross pathological changes should be recorded.

(6) MPCA enumeration in tissues, organs, and body fluids.

(i) Techniques. The presence of the MPCA in tissues, organs, and body fluids should be assessed by using sensitive, quantitative techniques, in those animals that die during the study, and in all animals that survive, at termination of the dosing period. Recovery values and detection and sensitivity limits should be determined and reported for each quantitative enumeration technique used.

(ii) Tissues, organs, and body fluids. The MPCA should be enumerated from the kidney, brain, liver, lung, spleen, blood, and representative lymph nodes. Other tissues, organs, and body fluids may have to be examined as indicated by the nature of any toxic and pathogenic effects observed.

(f) Data and reporting.

(1) Treatment of results. In addition to the information provided recommended by 150A-4, the test report should include the following information:

(i) the number of animals at the start of the test;

(ii) time of death of individual animals;

(iii) number of animals displaying other signs of toxicity and pathogenicity;

(iv) description of toxic and pathogenic effects;

(v) definition for one unit of the MPCA used, and the units/test animal in the dosing suspension;

(vi) body weights, food and water consumption;

(vii) necropsy findings;

(viii) pathology findings;

(ix) MPCA enumeration from tissues, organs, and body fluids, and methods used, and sensitivities and limits of detection, and;

(x) verification that each enumeration method is sufficiently sensitive to serve as a useful quantitative assay for the MPCA in tissues, organs, and body fluids.

(2) Evaluation of results. An evaluation should include the relationship, if any, between exposure to the test substance and incidence and severity of all abnormalities, including:

(i) behavioral abnormalities;

(ii) clinical abnormalities

(iii) gross lesions;

(iv) body weight changes;

(v) food and water consumption;

(vi) mortality;

(vii) toxicity; and,

(viii) pathogenicity.

(g) Tier progression.

(1) If significant or persistent signs of pathogenicity are observed in test animals, then consultation with the Agency is required for determination of further testing requirements. The ability of the MPCA to overcome natural host barriers to infection may be considered as a pathogenic trait, even when overt signs of disease are not apparent.

(2) If toxicity effects are observed, in the absence of significant pathogenic effects, then;

(i) the toxic component(s) of the dosing material are to be identified, and to a practical extent, isolated, and;

(ii) an acute toxicity study (152A-20) is to be conducted with the toxic components.

(3) If signs of infectivity, pathogenicity and toxicity are not observed, then no further testing is required. However, the registrant is required to provide an in-depth evaluation of possible consequences of unusual persistence of the MPCA in host organisms.

(4) If significant infectivity is observed in the absence of pathogenicity and toxicity, then a reproductive and fertility effects study (152A-30) is required.

Tier III

152A-30 Reproductive and fertility effects of microbial pest control agents [(MPCA)s]: Tier III.

(a) When required. Data from a one-generation reproduction and fertility study are required by 40 CFR 158.740 to support the registration of each end-use product that meets any of the following criteria:

(1) Significant infectivity of the MPCA is observed in test animals in the subchronic Tier II study (152A-21), and in which no signs of toxicity or pathogenicity were observed.

(2) The MPCA is a virus which can persist or replicate in mammalian cell culture lines (152A-19).

(3) The MPCA is not amenable to thorough taxonomic classification, but is related to organisms known to be parasitic for mammalian cells.

(4) The MPCA preparation is not sufficiently well purified, but it is indicated that the preparation may contain contaminants which are parasitic for mammals.

(b) Purpose. This guideline for a one-generation reproduction/fertility study is designed to provide information on the effects of a MPCA on fertility and on embryo/fetal development of test animals. Effects of the MPCA on fertility and fetal development to be evaluated include the number of non-pregnant females, numbers that gave normal birth, number of resorptions, litter size, delayed birth, embryolethality, and offspring body weight. Transmittal of the MPCA from parent to offspring also is evaluated.

(c) Principle of the test method. The MPCA is administered to male and female parents prior to their mating, and to maternal parents during the resultant pregnancies.

(d) Substance to be tested. Testing shall be performed with the technical grade of each active ingredient.

(e) Test procedures.

(1) Animal selection.

(i) Species and strain. The mouse or the rat are the preferred species. Commonly used laboratory strains should be employed. If another species is used, then justification/reasoning for the alternate selection should be provided. All test animals should be free of parasites or pathogens. Strains with low fecundity should not be used.

(ii) Age. Test animals should be between 6 and 8 weeks old prior to the first dosing.

(iii) Sex. (A) Both males and females are to be studied for an adequate assessment of the MPCA on fertility.

(iv) Numbers. Each test and control group should contain at least 20 males and a sufficient number of females to yield at least 20 pregnant females at or near term.

(2) Control groups.

(i) A concurrent untreated control group is required.

(ii) A separate vehicle control group is not required except when the toxicity of the vehicle is unknown.

(iii) A control group dosed with inactivated MPCA is recommended.

(3) Dosing. (i) Dose level. One dose level of at least 10^8 units of the MPCA per test animal should be used. Justification/reasoning must be provided if a dose level of at least 10^8 units test animal is not used. Quantification of the units of the MPCA should be done concurrently with dosing.

(ii) Dose route. Administration of the MPCA usually will be by the oral route. If persistence or infectivity of the MPCA in the Tier I studies was observed only after some other route of dosing (e.g. intravenous), then this route must be used.

(iii) Dose frequency. The frequency of dosing with at least 10^8 units/ test animal should be such that a significant level of MPCA is maintained in the parents prior to and during the mating period, and in the female parents during pregnancy.

(4) Observation period. Duration of observation should be from the initial dosing with the MPCA to sacrifice of the offspring.

(5) Observation of animals.

(i) A careful clinical examination should be made on each test animal at least once each day.

(ii) Additional observations should be made daily with appropriate actions taken to minimize loss of animals to the study, e.g., necropsy of, and MPCA enumeration from those animals found dead, and isolation of weak or moribund animals.

(iii) Cageside observations should include, but not be limited to, changes in:

(A) the skin and fur;

(B) eyes and mucous membranes;

(C) respiratory system;

(D) circulatory system;

(E) autonomic and central nervous system;

(F) somatomotor activity, and;

(G) behavior pattern.

(H) Particular attention should be directed to observations of tremors, convulsions, diarrhea, lethargy, salivation, sleep, and coma.

(iv) Individual weights of animals should be determined shortly before the test material is administered, weekly thereafter, and at death or at sacrifice.

(v) The time of death should be recorded as precisely as possible.

(6) Mating procedure.

(i) Parental

(A) For each mating, each female should be placed with a single, randomly selected male until pregnancy occurs or three weeks have elapsed. Mixed matings with other males should be avoided.

(B) Those pairs that fail to mate successfully should be evaluated to determine the cause of the apparent infertility. This may involve such procedures as additional opportunities to mate with other sires or dams, examination of the reproductive organs, and examination of the estrus or spermatogenic cycles.

(C) Each morning, the female should be examined for presence of sperm or vaginal plugs. Day 0 of pregnancy is defined as the day vaginal plugs or sperm are found.

(ii) Special housing. Near parturition, pregnant animals should be caged separately in delivery or maternity cages and provided with nesting materials. The cages and materials should be free from contamination by the MPCA. Dosing should cease prior to isolation of the pregnant females.

(7) Observation of pregnant females.

(i) Food consumption and prolonged parturition should be recorded, in addition to the above clinical observations (5i-v).

(ii) The duration of gestation should be calculated from Day 0 of pregnancy.

(iii) Each litter should be examined as soon as possible after delivery for the number of pups, stillbirths, live births, and the presence of physical and behavioral abnormalities.

(iv) Litters should be weighed at birth or soon thereafter.

(8) MPCA enumeration in parents and offspring.

(i) Infectivity or persistence should be assessed by using sensitive techniques to determine, as quantitatively as possible, the presence of the MPCA in test animals.

(ii) Organs, tissues, and body fluids of each male parent should be assayed at the time when it is confirmed that the female of the mated pair is determined to be pregnant.

(iii) Organs, tissues, and body fluids of each female parent should be assayed as soon as possible after birth of the litter.

(iv) Quantitative determinations of the MPCA in the pups from each litter should be made on postpartum day 1.

(f) Data and reporting. (1) Evaluation of study results.

(i) An evaluation of test results shall include a reporting of any and all effects of the MPCA on the test animals, all observations made, statistical analyses, MPCA quantification in the dosing preparations and in the parents and offspring, evidence that a significant level of the MPCA was maintained in the parents, dosing schedule, and animal weights. This should include an evaluation of the relationship, or lack thereof, between carriage of the MPCA by parents and abnormal effects on reproduction and fertility.

(2) Test report. In addition to the information recommended by 150A-4, the test report shall include the following information:

(i) fertility indices and length of gestation;

(ii) species and strain;

(iii) time of death during the study or whether animals survived to termination;

(iv) effects on reproduction and on offspring

(v) time of observation of each abnormal sign, including pathogenicity, and its subsequent course;

(vi) body weight data for parents and offspring;

(vii) necropsy findings;

(viii) MPCA enumerations; and,

(ix) statistical treatment of results, where appropriate.

(g) Tier progression. Any further testing that is to be required will be determined after consultation with the Agency.

152A-31. Oncogenicity study with microbial pest control agents [(MPCA)s]: Tier III

(a) When required.

(1) Data from oncogenicity testing may be required, as set forth in 40 CFR 158.740, to support the registration of each end-use product, or of each manufacturing-use product which legally may be used to formulate such an end-use product, when the potential for oncogenic effects in mammals is indicated by the presence of certain viral components of the product.

(2) A potential for oncogenic effects exists when a component of the MPCA formulation is a virus, and any of the following criteria are met:

(i) the virus is an intended or an unintended ingredient in the product and is known to be oncogenic for mammals, or is sufficiently closely related to such viruses.

(ii) the virus is an intended or unintended ingredient in the product and is demonstrated to transform mammalian cells in cell culture tests (152A-16); and,

(iii) efforts at characterization of a virus component of the product are insufficient to allow for the conclusion that the virus is potentially oncogenic.

(b) The study is not intended for evaluating the oncogenic potential of any product that may be due to chemical components, whether or not they are produced by a MPCA.

(c) Test standards. Since test standards will be developed on a case-by-case basis, consultation with the Agency is advised before performing an oncogenicity study.

152A-32. Immunodeficiency studies with microbial pest control agents [(MPCA)s]: Tier III

(a) When required.

(1) Data from immunodeficiency testing may be required to support the registration of each end-use product or of each manufacturing-use product which may legally be used to formulate such an end-use product, when the potential for causing a state of immunodeficiency in mammals is indicated.

(2) A potential for induction of immunodeficiency in mammals exists when a component of the MPCA is a virus, and the virus is, or is related to, any virus that is known to interact with components of mammalian immune systems and cause a state of immunodeficiency.

(b) Test standards. Since test protocols will be developed on a case-by-case basis, consultation with the Agency is advised before performing immunodeficiency studies.

152A-33. Primate infectivity/pathogenicity with microbial pest control agents [(MPCA)s]: Tier III

(a) When required. Data from primate testing may be required to support the registration of each end-use product or of each manufacturing-use product which legally may be used to formulate such an end-use product when:

(1) the potential for causing infectivity, pathogenicity, oncogenicity, or immunodeficiency is indicated by the presence of certain intracellular parasites in the pesticidal product.

(2) a potential for adverse effects in primates exists when a component of the MPCA formulation, during at least some stage in its life cycle, can be an intracellular parasite of mammalian cells, and any of the following criteria are met:

(i) A component of the MPCA formulation is a virus, and the virus is able to cause cytopathic effects in mammalian host cell lines in cell culture tests (152A-16) and, in addition, is demonstrated to replicate in mammalian host cells.

(ii) A component of the MPCA formulation is a known parasite of mammalian host cells.

(3) unresolved positive pathogenic effects from Tier I tests might be specific to the test animal(s) used.

(4) the taxonomic properties of the MPCA indicate that human pathogenicity might be of significant concern.

(b) Test standards. Since test protocols will be developed on a case-by-case basis, consultation with the Agency is advised before performing this study.

Series 153A: RESIDUE ANALYSIS GUIDELINES FOR MICROBIAL PEST CONTROL AGENTS

153A-1 Overview.

(a) Requirements. A petition for a tolerance or for an exemption from the requirement of a tolerance must be submitted as specified in 40 CFR 158.740 in connection with each application for registration of a microbial pest control agent (MPCA) where usage may result in residues in or on food for humans or feed for domestic animals used for human food. This petition must contain data satisfying the requirements of 40 CFR 158.740 which are detailed in this section series (153) unless specifically exempted from the requirements.

(b) Purpose. Residue chemistry data are designed to provide the information necessary to determine the site, nature, and magnitude of residues in or on food or feed. This information includes plant metabolism data, residue data, analytical methodology, and, when indicated, animal metabolism data and animal feeding studies to determine the carry over of residues into meat, milk, poultry, and eggs.

(c) Authority. Pesticides including MPCAs, intended for use on food or feed crops, or where usage may reasonably be expected to result (directly or indirectly) in residues in food or feed, will not be registered unless a tolerance, or an exemption from the requirement of a tolerance, has been established by the Agency, as provided for under Sections 406, 408, or 409 of the Federal Food, Drug, and Cosmetic Act ("FFDCA" 21 U.S.C. 346, 346a and 348). The procedural regulations for filing petitions for a tolerance or an exemption are included in 40 CFR 180.7.

(d). Approach. The use of a microbial pest control agent on food, feed, or raw agricultural commodities requires that a tolerance, or an exemption from the requirement for a tolerance, be established by the Agency. In considering exemptions from the requirement for tolerances, the Agency recognizes that MPCAs do not necessarily pose the same potential hazards as conventional chemical pesticides. In fact, certain characteristics of many of these agents suggest that they may pose relatively less hazard. These characteristics are listed below:

(1) The efficacy of the agent often depends upon its ability to replicate in the target pest, which is not likely to remain on the crop after harvest.

(2) The living form of the agent in most instances will usually not replicate in the absence of the specific target pest (e.g., insect host).

(3) Certain environmental conditions such as sunlight, rainfall, winds, humidity, and temperature often greatly reduce the viability of the agent; therefore, the residues of living organisms are apt to be small or relatively insignificant shortly after application.

(4) Data supporting currently registered MPCAs indicate that microbial pest control agents would not likely pose a hazard to humans or other mammals.

(5) In many instances where and when a microorganism is used as a microbial pest control agent, the microorganism is already normally present in the environment and has demonstrated no adverse effects.

(6) Residues of microorganisms used as microbial pest control agents that are capable of replication on food or feed--a very remote possibility--will possibly be rendered nonviable or be removed by the usual processing of such foods and feeds (i.e., washing, drying, heat sterilization, and additions of sugar, salt, and other preservatives).

(e) Tier Progression. The Agency evaluates residue data for microbial pest control agents used on food, feed, or raw agricultural commodities only if toxic or other harmful properties were observed in the maximum hazard toxicology tests (Tier I) prescribed in 152A-10 through -19 of this subdivision. If Tier I toxicology tests indicate no toxic or other harmful properties, then no residue data (with the general exception of a monitoring method) would be indicated and thus a recommendation for an exemption from the requirements of a tolerance can be made.

(f) Major Issues. In many cases, a natural population of microbial agent may be present at some background level at the site where a microbial pest control agent is applied. It may therefore be impossible to distinguish between natural and introduced microbial populations and therefore be very difficult to establish and enforce tolerances for naturally occurring MPCAs. The Agency invites comment concerning the testing methods for establishment and enforcement of tolerances for naturally occurring MPCAs.

153A-2 [Reserved]

153A-3 General residue data requirements for microbial pest control agents.

(a) When required. Residue data are required by 40 CFR 158.740 to be included in a petition for a tolerance or for an exemption from the requirement of a tolerance, in connection with each application for registration of a manufacturing-use product or end-use product composed of or containing an MPCA, when the following conditions are met:

(1) When the product is intended for use on food or feed crops; or

(2) When use of the product is expected to result in residues in or on food or feed; and

(3) When results of Tier I toxicology studies conducted in accordance with 152A-10 through -19 of this subdivision indicate that there may be significant human health concerns.

(4) Residue data will not be required and an exemption from the requirement of a tolerance will be recommended for products intended for use on food feed crops or for uses expected to result in residues in or on food or feed, when the toxicology data developed from Tier I testing, in accordance with 152A-10 through -19 of this subdivision, indicate that there are no significant human health concerns. The exception to this is that a monitoring method will be required for each registered MPCA, even if exempt from tolerance.

(b) Procedures, standards, and reporting. In addition to the provisions set forth in 150A-3 and -4 that are applicable, the following guidance paragraphs are provided for conducting, developing, and reporting the residue data that the Agency requires to support a petition for a tolerance or for an exemption from the requirement of a tolerance. In general, the guidance in Subdivision O (Series 170), particularly in terms of rationale and approach, is applicable to MPCAs under this subdivision. Unless addressed below, all paragraphs of Subdivision O as written are to be considered applicable to this subdivision; the term "pesticide" is assumed to include MPCAs. Discussion with appropriate Agency scientists may be helpful before steps are taken to develop residue data of the nature outlined in this series.

153A-4 Chemical Identity

In addition to the information detailed under 171-2 of Subdivision O, the MPCA must be identified and characterized. If the MPCA is a genetically altered microbe, then the nature and source (if applicable) of the inserted or deleted genetic material must be provided. The number of viable, infectious, or replicative entities (propagules) per unit weight or volume of product must be specified as well as the density of the product if it is a liquid.

153A-5 Directions for Use

In addition to the guidance under 171-3 of Subdivision O, the number of propagules per acre proposed for use is to be specified.

153A-6 Nature of the Residue in Plants

(a) When required. Plant metabolism studies are required to determine the nature of the MPCA residues in plants whenever an MPCA use is determined to be a food use. If a use is likely to result in MPCA residues in or on food, then this is considered to be a food use. In some cases, however, it may be possible to waive the requirements for plant metabolism data if no potential MPCA residues are expected to be of toxicological concern; such a waiver is most likely to be granted for an indigenous microbe that does not produce or code for a toxin. Attempts must be made to characterize all MPCA residues in or on plants regardless of their route of entry and/or deposition or whether they were produced via metabolic or strictly physicochemical processes.

(1) Potential residues of concern may be, but are not limited to, the following:

(i) all types of propagules of the parent (active ingredient) MPCA such as vegetative cells, sexual and asexual spores, virions, viroids;

(ii) mutants of the MPCA in question;

(iii) the genetic material itself;

(iv) any antigenic /allergenic, toxic, and/or mutagenic substances associated with the MPCA product either as impurities in the formulation or as components or metabolic products of the MPCA itself produced during the manufacture or storage of the product or at the site of application;

(v) or any other replicating entity which is the recipient of MPCA genetic material (chromosomal or extrachromosomal) of potential concern (such recipients may be viruses or cells of animals, plants, or bacteria). The genetic stability (ease of genetic exchange or transfer) is thus important in determining potential "residues" of concern, especially in the cases of nonindigenous or genetically altered MPCAs.

(b) Test procedures and reporting of data. The uptake, metabolism, and translocation of the MPCA must be determined, typically in a representative member of each crop group defined in 40 CFR 180.34(f) except the Herbs and spices group. If the metabolism is similar in three unrelated crops, then additional crops normally need not be included. In the case of demonstrated or potential plant pathogens, however, more extensive testing may be required, especially since replication of the MPCA may occur in nontarget crop species; consultation with appropriate Agency scientists should be sought if a petition involves a plant pathogen. If possible, treated plants must be grown to normal crop maturity so that residues may be characterized in or on the raw agricultural commodities (RACs) derived from the crop in question (listed in Table II of Subdivision O).

Plants must be treated via the proposed route (seed treatment, soil treatment, foliar plus soil treatment, etc.). Since intentionally added inert ingredients could significantly influence MPCA deposition, viability/stability, absorption, and even replication at the treatment site, the test substance should be a typical end-use product (EP). In some cases, radiolabeling of the MPCA may be useful but, generally, other approaches must be employed to determine the total terminal residue and, subsequently, which terminal residues are of toxicological concern (refer to Series 152 of this subdivision for guidance). The application rate should be the maximum proposed rate or exaggerated rates, if necessary for residue identification.

MPCAs, being much more complex than conventional chemical pesticides, create unusual analytical problems. For example, the total terminal residue may consist of drastically different entities (whole microbes, toxins, etc.) each requiring greatly differing analytical procedures. Also, since all or most MPCAs are replicating entities, the total terminal residue will frequently increase with time but not as a function of continued residue absorption and/or transformation of one residue to another as is typically the case with conventional pesticides. In some cases, the viable MPCA per se may not be of toxicological concern but a toxin produced by the MPCA may be of concern. Note, however, that the MPCA itself will be regulated even if it serves only as a source of a residue of toxicological concern.

Some MPCAs may remain in the soil and/or on aerial plant parts whereas others may gain entrance into plants via active or passive routes. Once in the plant, the MPCA may be transported to other plant parts, may replicate, and may cause disease; in other cases, replication may occur only at the site of entrance. Replication may occur only in arthropod or other hosts in or on plant tissues. In some cases, the MPCA may be restricted to the soil or an isolated plant part, perhaps not a RAC, but exert its influence throughout the plant such as in the case of a translocatable toxin. MPCA residues may fluctuate with the growth cycles. It is the responsibility of the petitioner to demonstrate which, if any, plant parts bear which residues of concern, i.e. to determine the distribution of residues. These studies will demonstrate which are the major residues to be sought in field residue studies, if required, and also will provide an indication as to the efficiency and sensitivity of the methods used. Refer to 153A-8 for a discussion of some analytical methods expected to be of use for MPCA determination.

153A-7 Nature of the Residue in Animals.

(a) When required. Animal metabolism studies are required to determine the nature and distribution of the MPCA residues in animals if the following conditions are met:

(1) MPCA residues have been determined to be of toxicological concern, i.e. Tier II or III toxicology testing must be performed (152A-20 through -32 in this subdivision): and

(2) Residues of concern will be present in or on feed items (see Table II of Subdivision O); or

(3) Direct animal or animal premise treatments are proposed. These types of studies may be required even if no residues of toxicological concern occur in or on feed items because animal and plant "metabolism" may be different.

Waivers of these data requirements are possible if the petitioner is able to adequately demonstrate that no residues of toxicological concern will occur in animal products used for food. Refer to 153-6(a)(1) for examples of potential residues of concern.

(b) Test procedures and reporting of data. If one or more of the conditions above are met, then animal metabolism studies must be conducted utilizing ruminants and poultry. If residues of toxicological concern occur in or on feed items, then animals generally must be dosed orally for at least 3 days with the MPCA terminal residues in feed items characterized to the extent possible. Note that, theoretically, a single MPCA propagule in or on a feed item could cause infection or allow replication in livestock; obviously, this single viable unit could be a residue of toxicological concern but would most likely not have been detected in or on the feed item. Therefore, if any potential for animal pathogenicity exists, animal metabolism studies will be required in all cases. If orally or dermally administered direct animal treatments or animal premise treatments are proposed, then animals/premises must be treated according to the proposed use directions or at exaggerated rates (if necessary for residue characterization) using a typical EP product.

If no replication of the MPCA occurs in or on the animal, then at least muscle, fat, kidney, and liver must be analyzed within 24 hours of dosage cessation. If MPCA replication occurs in the animal, then several longer intervals may also be required and more extensive tissue sampling will be necessary. Some MPCAs, notably fungi, may require weeks or months to establish a detectable infection. Eggs and milk, where applicable, must be sampled at regular intervals, preferably daily. If may be possible to combine the animal metabolism and animal magnitude studies (153A-10 of this subdivision), if required. These studies will allow the determination of the major residues for which analytical methodology must be developed and those which must be sought in animal feeding studies, if required. The efficiency and sensitivity of the utilized methods will also be determined from these animal metabolism studies.

153A-8 Residue Analytical Methods.

Analytical methods are required both for data collection to support proposed tolerances and for the enforcement of such regulations (40 CFR 180). Note that a monitoring method is required for the determination of all MPCAs that are exempt from the requirements of tolerance; the Agency must have this monitoring method available in times of need and cannot afford the potentially long method development period in the event adverse effects are observed subsequent to registration. The method(s) must not be subject to interference due to substrate, reagents, or residues (cells, virions, toxin, etc.) of related or unrelated microbial agents whether naturally occurring and unregistered or whether an MPCA. A confirmatory procedure is also required for each residue of concern for both data collection and tolerance enforcement.

Each method must be fully described or a reprint must be provided as well as any necessary modifications. Each method must be validated by submitting recovery data and analyses of untreated control samples of representative plant and animal commodities. The estimated sensitivity/detection limit must be provided for each tested commodity. Attempts must be made to determine if residues in or on treated commodities (aged or weathered) are extracted with the same efficiency as those from spiked samples used for recovery experiments.

Widely differing analytical methods may be required to identify and quantify all residues of toxicological concern derived from a given MPCA. If a biologically active microbial product is of concern, then the more conventional analytical procedures such as gas chromatography, mass spectrometry, or high-pressure liquid chromatography are typically used. If the MPCA per se, a mutant, or a viable recipient of MPCA genetic material is a "residue" of toxicological concern, then various immunological methods (such as enzyme-linked immunosorbent assay, dot-immunoassay) or molecular probe methods (such as dot hybridization, Southern hybridization procedure, or restriction endonuclease mapping) may be used for identification and/or quantification. Since the above procedures do not necessarily determine viable MPCAs, culturing of tissues (maceration followed by dilution plating) or infectivity assays will frequently be necessary. Culturing or bioassays are also important as means of detecting MPCAs at levels below the detection limits of the above methods and, theoretically, as few as one viable microbe can be detected using enrichment techniques, if necessary. In some cases, microscopy may be useful.

It may be possible to purify some viral MPCAs to the point of crystallization whereas other MPCAs may not be isolatable from host cells or host membranes in a viable form. Some MPCA residues may be bound (actively or passively) to cellular structures/components and their release must be attempted using procedures such as sonication, use of detergents, or hydrolytic steps (enzymatic, acid, or alkaline). Care must be taken to determine background levels of cross-reacting MPCAs since antigenic similarities and/or nucleic acid homology may exist in indigenous microbes to a greater or lesser extent. In some cases, care must also be taken to detect different viable forms of the MPCA in question (such as spores vs. vegetative cells, encapsulated vs. nonencapsulated, or "yeast" vs. mycelial forms) since antigenic determinants may be different or may be masked. In the case of genetically altered MPCAs, the methods must be specific enough to determine the MPCA in the presence of the parent, unmodified, indigenous strain of the same microbe which, generally, will differ only in a relatively small portion of nucleic acid (chromosomal or extrachromosomal).

The regulatory method(s) must be relatively simple, rapid, specific, and sensitive and should not require blank samples, exotic equipment or reagents, or use of internal or procedural standards. If the Agency finds the regulatory method(s) adequate, it (they) will be published or referenced in the FDA Pesticide Analytical Manual after an exemption from tolerance or a permanent tolerance has been established.

153A-9 Storage Stability Data.

Storage stability data, as described in 171-4(c)(1)(ii) of Subdivision O, must be submitted. Studies involve spiking representative plant and animal samples with each "residue" of concern, storing under the same conditions (usually subfreezing temperatures) as the treated samples, and analyzing at the end of the treated sample storage period. It is imperative that samples be stored at temperatures sufficiently low enough to prevent replication of the MPCA or production of biochemicals of concern. Note that freezing may be injurious to some MPCAs. If this is the case, very short harvest-to-analysis intervals must be used or other storage conditions must be devised.

153A-10 Magnitude of the Residue in Plants.

MPCA residues of toxicological concern must be determined in or on RACs of all crops on which use is proposed or, if preferred, RACs of each representative crop belonging to a given crop group under 40 CFR 180.34(f) to allow the establishment of a crop group tolerance. If residues are detectable in or on the RAC or if concentration in processed products is possible, then processing studies will also be required. In general, the procedures described under 171-4(c) of Subdivision O should be used for guidance making certain that a representative of each major formulation class is tested according to the proposed use pattern. Table II of Subdivision O lists the RACs and processed products of each agricultural crop. Note that "pesticide" and "chemical" in Subdivision O are assumed to include MPCAs for purposes of this subdivision. Residue increases and/or decline in : on crops will frequently be a function of growth or replicatn cycles of the MPCA which, in turn, is a function of the population namics of potentially numerous other organisms such as the host, e plant site (if different from the host), other parasites/paogens of either the host or the MPCA itself, or competing occupantof the same or a similar habitate. It is expected that enviroental conditions will play an even greater role in the magnitude oMPCA residues in or on plants than in the case of conventional chical pesticides. Therefore, adequate geographic representation of st sites is imperative; refer to USDA's Agricultural Statistics (O), published annually, for the most recent state production fures for various crops. Note that Subdivision O [171-4(c)] statethat a processing study utilizing radiolabeled material may be reired if processing of the RAC could result in alteration of the sidue; in the case of an MPCA, radiolabeling will rarely be eitr possible or of utility and, therefore, other approaches must be sed.

In addition tche above information and the residue data itself, the follow materials and procedural details must be provided: location test, application rate (weight or volume of product/A and numbeof viable microbes/A, etc.), formulation used, part of crop analyd, number of samples, sampling procedure, planting time (date), apication date(s), harvest/sampling date(s), application method,tage of crop at application and harvest, analytical method useduntreated control data, recovery data, and sample storage timend conditions.

153A-11 Magnitude the Residue in Animals.

Generally, he guidance presented in 171-4(c)(3) of Subdivision O is apicable to MPCAs under this subdivision noting that, again, "pestide" is assumed to include MPCAs. If residues of toxicological coern occur in animal tissues, milk, or eggs following oral dosi in the animal metabolism studies, then feeding studies are requirereflecting 1x, 3x, and 10x the maximum expected dietary intake of MA residues occurring in or on feed items. Feed items derived om each RAC are listed in Table II of Subdivision O. Animals must gerally be dosed for 28 days and slaughtered within 24 hours of e final dose. If pathogenicity, toxicity, or very slow growth anor disease development is a problem, then appropriately short or longer feeding periods or perhaps even longer preslaughter ntervals may be used, preferably in consultation with Agency scientis. The key issue is timing, i.e. the maximum residue/MPCA concentations in tissues, milk, and eggs must be determined in order allow the tolerances and, perhaps, preslaughter intervals, to be estlished. Similarily, if residues occur in animals following a rect animal treatment (if proposed) as determined in an anil metabolism study, then studies utilizing typical EPs must be nducted according to the proposed use directions; such treents could be feed-through, dermal, or otherwise. If two or more routes of exposure are possible, then a single study combining both routes are acceptable if the health of the animals is not affected. Note that the petitioner may find it advantageous to combine the animal metabolism studies (153A-7 of this subdivision) and the feeding/direct animal treatment studies.

The dosage rate must be clearly stated. In the case of a feeding study, ppm of nonviable residues and enumeration of viable residues (colony- or plaque-forming units or infectivity units/unit weight of feed or per unit weight per day) as well as the weights of animals must be provided. In the case of direct-animal treatments (oral, dermal, or otherwise), the weight or volume of product (typical EP) and the number of viable MPCAs per unit weight, surface area, etc. of animal must be provided. Generally, ruminant and poultry studies are required if residues of toxicological concern occur in feeds or if direct animal treatments are proposed. Swine studies may also be required if there is any reason to expect higher residues and/or more rapid MPCA replication in swine than in poultry or ruminants. If agricultural premise treatments are proposed, then studies may be required to demonstrate the magnitude of the residue in animals following these uses. All tissues used as food must be sampled; eggs and milk must be sampled twice daily. Sample storage time and conditions must be provided as well as the identity of the analytical methods used, recovery data, and control animal data. Also, the formulation used and number of samples must be presented.

153A-12 Potable Water, Fish, and Irrigated Crop Studies.

The intent and guidance provided under 171-4(c)(4) of Subdivision O are generally applicable to MPCAs proposed or registered for use in or near aquatic sites. Note that it is possible that replication of the MPCA may occur in irrigation water, natural waterways, fish (including crustaceans), or irrigated crops. Therefore, rather than dissipating or becoming diluted, MPCA residues of toxicological concern may actually increase in the new environment. New metabolites of concern may actually form. Note that a typical EP should be used rather than the ^{14}C-labelled material described in 171-4(c)(4) for the fish metabolism study, if required.

153A-13 Food Handling Establishment Studies.

Refer to 171-4(c)(5)of Subdivision O for guidance useful in developing studies to support use of MPCA products in food handling establishments.

153A-14 Practical Methods for Removing Residues That Exceed Any Proposed Tolerance (Section E of a Petition).

Refer to 171-5 of Subdivision O for guidance.

153A-15 Proposed Tolerances (Section F of a Petition).

Refer to 171-6 of Subdivision O for guidance.

153A-16 Reasonable Grounds in Support of the Petition (Section G of a Petition).

Refer to 171-7 of Subdivision O for guidance.

153A-17 Exemptions from the Requirement of a Tolerance.

Refer to 171-8 of Subdivision O for guidance.

153A-18 Tolerances for Foreign Uses.

Refer to 171-9 of Subdivision O for guidance.

153A-19 Rotational Crop Tolerances.

Refer to 171-10 of Subdivision O for guidance. If viable MPCAs remain in soil for 18 months or more or if the MPCA in question replicates in one or more rotational crops or their associated flora or fauna, then residue data must be collected as described under 153A-10 and 171-10 of these guidelines.

153A-20 Tobacco Uses.

Refer to 171-11 of Subdivision O for guidance.

153A-21 Data Requirements for Food Use vs. Nonfood Use.

Generally speaking, data will be required to demonstrate that a given use on a food crop (seed treatment, grown only for seed, fallow land, or nonbearing crop) is not a food use. Since MPCAs are replicating entities, there are very few uses, if any, which could not ultimately be food uses. Refer to 171-12 of Subdivision O for guidance.

153A-22 Submittal of Analytical Reference Standards.

Refer to 171-13 of Subdivision O for guidance.

153A-23 Special Considerations for Temporary Tolerance Petitions.

Refer to 171-14 of Subdivision O for guidance concerning residue chemistry data requirements needed to establish a temporary tolerance in conjunction with an Experimental Use Permit (EUP).

153A-24 Presentation of Residue Data.

Refer to 171-15 of Subdivision O for guidance.

153A-25 Translation of Data.

Refer to 171-16 of Subdivision O for guidance.

Series 154A: NONTARGET ORGANISM HAZARD GUIDELINES

154A-1 Overview

The purpose of nontarget organism testing is to develop data necessary to assess potential hazard of microbial pest control agents (MPCAs) to terrestrial wildlife, aquatic animals, plants, and beneficial insects.

(a) Approach. The Agency has concluded that at least some test data on terrestrial and aquatic organisms should usually be evaluated, regardless of the pesticide's site of outdoor application and apparent potential for exposure. These data would be necessary for the following reasons:

When a microorganism is applied as a pesticide, great numbers are placed in the environment apart from its host, at a discrete point in time (day of application), and spread over living and nonliving components of the target site. Often, there will be spread to adjacent areas, due to drift. Hence, in terms of numbers of nontarget organisms exposed, number of different species exposed, and the degree of exposure (number of microorganisms per nontarget organism), exposure may be greater than under natural conditions. In addition, data on toxic or pathogenic effects are essential for hazard assessment purposes when terrestrial or aquatic organisms are likely to be exposed to a MPCA, especially when no fate data will be required by the Agency in the first tier of testing.

Pathogenicity and toxicity appear to be the major effects of concern regarding exposure of terrestrial and aquatic organisms to microbial pesticides. Therefore, the Agency has developed guidelines that will allow hazard assessment of pathogenicity and toxicity problems to be made. The Agency desires a high level of confidence that no unreasonable adverse environmental effects will result from actual use of MPCAs. Toward this end, the guidelines in Tier I reflect a maximum hazard approach to testing. Negative results from tests using this approach would provide a high degree of confidence that no unreasonable adverse effects are likely to occur from the actual use of MPCAs.

If unacceptable adverse effects are identified in Tier I tests, then Tier II tests are performed to attempt to quantify levels of the MPCA to which the susceptible nontarget species may be exposed. Prior to registration of MPCAs, applicants would submit Tier I data on nontarget organisms. However, environmental expression data (Tier II) may also be required on a case-by-case basis for certain MPCAs which are determined to present unique concerns. In addition, on a case-by-case basis, definitive Tier II data showing that the MPCA will not survive or persist in the environment to which it is applied, can be submitted as support for a request for waiver (40 CFR 158.45) of some or all of Tier I testing requirements. In some cases, a subchronic test may serve to better understand the effects observed at the Tier I level and might alleviate the need for Tier II testing.

If the MPCA is found to persist or survive in the environment at significant levels as shown by Tier II tests, Tier III studies are designed to show effects of chronic exposure to these levels on fish and wildlife. If it is indicated that there may still be a problem, Tier IV studies (simulated or actual field studies) may be able to determine if there is a problem under actual use conditions.

(b) Major issues.

(1) Maximum hazard dosage levels. Unlike environmental levels of chemical pesticides, which generally decrease following application, the environmental levels of MPCAs and any associated toxins may, at least temporarily, increase when the product is effective. Therefore, the maximum hazard dose for Tier I testing will be based on some safety factor times the maximum amount of active ingredient (MPCA or its toxin) expected to be available to terrestrial and aquatic plants and animals in the environment. The target hosts (e.g., insects) are likely to contain the highest concentration of the microbial pest control agent that will be available to nontarget terrestrial wildlife and aquatic animals following a pesticide application.

Avian wildlife will be exposed, most commonly, through the diet (via infected insects) or through the respiratory tract (via spray drift or aerosolization). The maximum amount of MPCA a bird in the wild may consume is difficult to determine, but as much a 1×10^9 is possible. Due to anatomical constraints, the Agency recognizes that dosing at this level can not always be achieved. Thus, the recommended daily oral or injected dose should be calculated as follows:

$$\text{Max. daily dose (units)} = \text{MPCA Concentration in TGAI} \times \text{5 ml/kg Body Weight} \times \text{Weight of Test Bird (kg)}.$$

Therefore, for a product whose TGAI contains 1×10^9 units/ml and using a 25 gram bobwhite quail, the maximum daily dose would be:

$$(1 \times 10^9 \text{ units/ml})\ (5 \text{ ml/kg})\ (0.025 \text{ kg}) = 1.25 \times 10^8 \text{ units of MPCA}$$

This dose should be administered over a 5 day period so that the total dose the bird would receive orally, over a five-day period, would be 6.25×10^8 units.

Maximum doses for the respiratory administration should be calculated in a similar manner except that the dosing volume should be reduced from 5 ml/kg to 0.2 ml/kg.

Maximum hazard aquatic exposures must, in some way, account for the fact that fish and aquatic invertebrates, are less mobile than terrestrial species and, therefore, less able to avoid the pesticide. In addition, under conditions of nutrient influx or the presence of alternate hosts or target pests in aquatic ecosystems, aquatic organisms may be exposed to elevated numbers of microbial pesticides. It is recommended, then, that the maximum hazard exposure be in the range of 1×10^6 units of MPCA per ml of water or in a concentration 1000 times the calculated concentration after direct application to a 6-inch layer of water at label rates if the microorganism produces a toxin. Aquatic exposure will simultaneous expose fish by the dietary route.

The Agency realizes that it would be very difficult to establish specific LC_{50}, ED_{50}, or LD_{50} values (e.g., $LD_{50} = 1000$ mg/kg) and 95 percent confidence limits for most microbial pest control agents whose mechanism of action is pathogenicity, because test data are not likely to exhibit a log-probit dose-response relationship that is typical of chemical pesticides. Therefore, data that establishes an LC_{50}, ED_{50}, or LD_{50} that is greater than the maximum hazard dosage level (e.g., LD_{50} >1000 mg/kg) would often be adequate for the purposes of hazard assessment. In most cases, testing at one maximum hazard dosage level is expected to be sufficient to evaluate effects for these MPCAs. MPCAs that are toxin-producing are more likely to produce a log-probit response. Therefore, in most cases multiple groups would be necessary in order to quantify the hazard of these organisms. If there are no effects at the maximum hazard dose, lower doses will not be necessary.

(2) Maximum hazard routes of administration. Various routes of administration (dosing) are provided for in these guidelines and are chosen to reflect "natural" exposure routes. The Agency believes that these routes; oral and respiratory for birds, aquatic and food exposure for aquatic organisms, and the oral route for insects can best define the hazard to nontarget organisms in the wild.

Parenteral dosing, such as intravenous and intraperitoneal injection, would provide a high degree of confidence that a particular microbial pesticide would not cause adverse effects, if negative. Positive results, on the other hand, given the complex and undefined (exogenous protein, metabolic byproducts, etc.) components of microbial pesticide preparations and the environmentally unrealistic nature of the route, would be difficult to translate to effects on species in the environment. However, due to the high degree of confidence an injection test gives, it is being suggested as an alternate exposure route in the Avian Acute Oral, Toxicity/Pathogenicity Test whenever the microbial dosing prepation is sufficiently free from exogenous protein and other contaminating substances so that the test will not be confounded.

(3) Age of the test animals. The Agency considers that sufficient immunological and physiological differences exist between immature animals and mature animals to suggest that immature animals

are potentially more susceptible to infection and possibly to the effects of any toxin produced by the MPCA. Therefore, the Agency has developed age guidelines for the test animals in Tier I tests, and recommends the use of immature animals in keeping with the maximum hazard approach to testing.

(4) Methods for detecting the microbial pest control agents. Unlike toxicity tests where mortality can usually be determined by observation, infectivity tests often require sophisticated assessment methods for detecting sublethal pathogenic effects. These methods may include serological or nucleic acid technology.

(5) Detailed test protocols. No standard, widely accepted, laboratory validated, test protocols are available at this time to evaluate the safety of microbial pest control agents to terrestrial and aquatic animals. The EPA Office of Research and Development is in the process of developing and validating detailed testing protocols for the Office of Pestice Programs (OPP). In the meantime, the draft and final protocols, as they become available, may be obtained on request from the Environmental Fate and Effects Division of OPP.

(6) Length of tests. The guidelines provide that the duration of all Tier I tests be about 30 days long. This should permit time for incubation, infection, and manifestation of effects in the test organisms for most microbial pest control agents. Some test species, notable nontarget insects, may be difficult to culture and the test duration has been adjusted accordingly. Recommended test durations are included for each testing guideline.

Various authors have proposed test duration times for toxicity and pathogenicity tests ranging from 14 to 35 days (Ignoffo 1973; Ignoffo et al. 1975; Summers 1975). The Agency realizes that the test duration period may be unnecessarily long, or may not be sufficiently long enough to detect effects such as viral diseases that recur after prolonged intervals of latency, e.g., Herpes zoster (Fenner et al. 1974). At the present time, however, the Agency is not aware of an accurate method to predict whether a virus detected in a test organism will manifest latent effects. The Agency invites comments on the proposed test duration period and the probability of encountering MPCAs with latent effects.

(7) Control groups. Appropriate control groups are addressed in the recommended guidelines for each test.

154A-2 Terrestrial Wildlife.

(a) Approach. These guidelines call for two tests on birds for all MPCAs: an avian acute oral toxicity and pathogenicity test (154A-16) and an avian respiratory pathogenicity test (154A-17). The avian acute oral toxicity and pathogenicity test would provide data on any toxic effects to avian wildlife from exposure to the microorganism or any toxin it may produce. This test would also provide data on pathogenic effects following an acute exposure either by the oral (or injection) route. The duration of the study would be about 30 days to allow for an incubation period prior to onset of symptoms.

The avian respiratory pathogenicity test would provide data on the pathogenic effects of the MPCA on birds following exposure due to drifts or aerosolation. The guidelines for the duration of the test and gross necropsies are similar to the avian acute oral toxicity and pathogenicity test.

In both the acute dose and inhalation tests, gross necropsy, histopathological examination and culture and isolation should be performed on exposure site tissues and other organs showing anatomical or physiological abnormalities. In some cases, such as viruses, there is a preference for certain cell or tissue types. In cases where tissue preferences are known or suspected, those tissues should be examined whether or not gross anatomical or physiological changes are seen.

(b) Tier Progression.

(1) Tier I. If no toxic or pathogenic effects are observed after exposing birds to the microbial pest control agent via two different routes of administration (oral and respiratory) at the maximum hazard dosage levels, then no further testing of birds would be indicated. If toxic or pathogenic effects are observed at the maximum hazard dosage levels, then Tier II, environmental expression tests (155A), would be indicated. In some cases, a subchronic test may serve to better understand the effects observed at the Tier I level and might alleviate the need for Tier II testing.

Data on wild mammal toxicity and pathogenicity (154A-18) are required on a case-by-case basis when data indicate that there is considerable variation in the sensitivity of different mammalian species to the effects of a MPCA or where wild mammals would be heavily exposed to the MPCA under normal use. The toxicity and pathogenicity data in section series 152A of this Subdivision for evaluating hazard to humans and domestic animals are normally adequate to indicate hazard to wild mammals. If no toxic or pathogenic effects are observed in these tests, then no further testing of wild mammals would follow. If any effects are observed in tests on wild mammals, then Tier II, environmental expression testing (155A), would be indicated. In some cases, a subchronic test may serve to better understand the effects observed at the Tier I level and might alleviate the need for Tier II testing.

(2) Tier II. The data outlined in Tier II are described in the environmental expression testing sections (155A) of these Guidelines. If the expression characteristics preclude exposure of the MPCA to nontarget birds and mammals, then no further testing of these animals would be indicated. If Tier II tests indicate that birds and mammals will be exposed to the MPCA, then testing at Tier III would follow.

(3) Tier III. The types of effects reported in the Tier I tests would determine which Tier III test(s) would apply. If adverse effects are reported in Tier I tests, and Tier II tests indicate exposure, then Tier III testing would be required. If reproductive or fertility effects, or oncogenicity are reported in tests in 152A30, and -31 for evaluating hazards to humans and domestic animals, then a long-term avian pathogenicity and reproduction test (154A-26) would apply. This test would provide data on pathogenic effects of the MPCA on birds during a critical period in their life--breeding and reproduction. It would also provide data on the effects of the MPCA on avian reproduction. If no pathogenic or reproductive effects are observed, the Agency would, at this time, review all the data and determine if decisions regarding registration can be made.

Pathogenic effects occurring at Tier III and beyond raise serious questions concerning the registration of any MPCA. Also, testing at Tier IV, simulated and actual field testing for mammals and birds (154A-33) may not be feasible, since it may not be possible to confine the MPCA to a test area and prevent it from contaminating adjacent areas. In such a case, simulated field testing may be possible but would necessitate a very complex design.

(4) Tier IV. Simulated and actual field testing (154A-33) would provide data on the pathogenic effects of the MPCA on birds and mammals following field applications at actual label use rates. This test would be indicated when pathogenic effects are reported in Tier III testing (154A-26) at levels equal to actual or expected field residue exposure levels, and when the Agency is reasonably confident that quarantine methods will prevent MPCA dispersal to pen, large-pen, or full-scale field tests) should be discussed with the Agency before beginning the study. Protocols for such studies must be submitted to the Agency and must receive Agency approval prior to test initiation.

(c) Major issues for discussion. In the process of developing the guidelines for terrestrial animals, the Agency recognized many important areas that require outside input and comment. The Agency invites scientific input and comments on the following issues of concern:

(1) In vivo testing. The guidelines outline in vivo testing of birds and mammals. In vitro testing may be considered in the future. Wolf (1975) has suggested a two-pronged testing approach for safety testing of baculoviruses, using both in vivo and tissue culture testing. He reported that there are established or permanent cell lines for duck embryo fibroblasts, chicken embryo fibroblasts, and representative mammalian cell lines from a bat, rabbit, mouse, and deer. Ignoffo (1973) reported that at least 12 viruses, including all major viral types, have been tested in vitro in either avian egg embryo fibroblasts (chicken or turkey), fish, or mammalian cell lines. Virus multiplication or cytopathic effects were reported for one nuclear polyhedrosis virus in chicken embryo cells and human amnion tissue, and for one noninclusion virus in chicken embryo cells and mouse sarcoma tissue. In contrast, no effects were observed in vivo when rabbits and mice were injected or fed the latter virus. More recently, Brusca, et al., (1986) have shown that Autographica californica NPV can penetrate the nucleus of 3 poikilothermic vertebrate cell lines, although no productive infection was demonstrated.

The Agency is not convinced at this time that the results of in vitro tests can be used exclusively to determine potential adverse effects to individual terrestrial animals (e.g., endangered species) or populations of terrestrial animals in the environment.

(2) Test substance. Microorganisms used as pesticides could be applied in any one of a combination of naturally existing forms. It is preferable that the test organism be exposed to the most infectious form whenever infectivity is the primary hazard of concern. Similarly, when toxicity (e.g., a microbial toxin) is the hazard of concern, the test organism should be exposed to a form of the MPCA in which the toxin would be produced in the greatest amount and most readily available. Unfortunately, there is no easy way to determine which is the most infectious or toxic form of the microorganism to the test organisms. The route of administration may also play an important role in determining which form should be tested. For example, if the route of administration is intravenous, then the active vegetative cells of a bacterium, or the infectious hemolymph may be more appropriate than vegetative cells or polyhedryda, respectively.

For these guidelines, testing the technical grade of the active ingredient applies in all tests except the simulated and actual field testing (154A-33), where the use of the formulated product applies in order to simulate or reproduce actual field use. The Agency realizes that in some cases the technical grade of the active ingredient and the formulated product may be identical.

(3) Route of administration. These guidelines outline testing by oral gavage or by injection and via the respiratory tract. It is important to note that the administration of test material to 14- to 28-day old birds by oral gavage will likely require the use of small needles or cannulae with ball-tipped ends in order to prevent

injury to the birds. Ignoffo (1973) reported that the following groups of terrestrial animals have been tested in vivo for effects caused by entomopathogens:

Group	Routes of Administration
Mammals (primarily laboratory populations)	- Diet, oral, inhalation, subcutaneous, dermal application, intradermal, intraperitoneal, intravenous, intracerebral, intranasal, intramuscular, eye application.
Birds (chickens and laboratory populations that are phenotypically similar to wild species)	- Oral, diet, intraperitoneal (chickens).

Since the gut normally provides such a radically different environment from that in the rest of the bird or mammal body, and since insectivorous birds and mammals can be expected to ingest large quantities of actively growing microorganisms when they feed on diseased insects, the Agency believes that the oral route would be appropriate. The dietary route of administration was considered for Tier I tests, but the Agency believes that it does not generally reflect the maximum hazard test philosophy for Tier I tests. The diet, however, is considered to be an appropriate route of administration for Tier III and Tier IV tests (154A-26 and 154A-33).

The injection route may be used as an alternative route of exposure for the oral toxicity/pathogenicity test, if the microbial preparation does not contain excessive protein, metabolic byproducts, etc. This route, although not environmentally realistic, provides a maximum hazard challenge by bypassing the animals primary defense mechanisms. If an injection route of exposure is used, a single dose is acceptible in lieu of the multiple oral doses. Negative results obtained by this dosing method indicate, with a high level of confidence, that the MPCA under study will not produce adverse effects to exposed avian species.

Inhalation, or rather intranasal or intratracheal instillation has been chosen as the second exposure route because birds may be exposed by this route during spraying operations or by the MPCA made airborne through the effects of wind or animal movement during feeding or other activities. In addition, the respiratory tract is a major portal of disease aquisition in avian species.

The Agency is aware of the theoretical potential of microbiological pesticides to disrupt the function of rumen bacteria. At present, the Agency is seeking further information concerning the possibility of such effects on wild mammals. If any such effects were to be reported in safety tests on domestic ruminants, then the Agency would solicit similar tests on wild ruminants.

The Agency recognizes that a combination of administrations in one test (e.g., oral and intravenous or intraperitoneal injection) may be possible. It would certainly be in keeping with the maximum hazard testing philosophy and would reduce testing time and expense. However, combined exposures could unduly traumatize the test animals so as to cause mortality, or in some other way cause spurious results.

(4) Avian test species. These guidelines provide that young bobwhite quail or mallard ducks be tested in Tier I tests. Birds between 14 and 28 days of age at the beginning of the test period should be used in the avian oral toxicity and pathogenicity test and in the avian pathogenicity test. Within a given test, all birds should be the same age (U.S. Environmental Protection Agency 1978).

Summers et al. (1975) suggest testing two species of birds including at least one insectivorous species. Wolf (1975) stresses that test organisms should represent insectivorous and herbivorous species. He suggests testing blackbirds, yellow-billed cuckoos, representative members of the swallow family, and ducklings.

In Subdivision E of the Guidelines, the Agency suggests bobwhite quail, ringneck pheasants, and mallard ducks as acceptable test species for avian acute toxicity tests of chemical pesticides.

The following facts influenced the Agency's proposal to test bobwhite quail and mallard ducks in avian toxicity and pathogenicity tests of MPCAs:

These species are ecologically significant and widely distributed in the United States. They have proven to be good laboratory test species and are appropriate for acute, subacute, and chronic testing. Laboratory populations are comparable to wild species. There is a large amount of baseline necropsy and histological data available. It has not been determined if any avian species or group of avian species is a better indicator of potential effects from microbial pesticides than bobwhite quail or mallard ducks, and, finally, testing species from the family Icteridae (e.g., blackbirds, grackles, and cowbirds) may be ecologically significant and in line with the maximum hazard philosophy but would present many practical problems in rearing, reproduction, controls, and handling.

In support of testing immature birds in Tier I, the Agency notes that insects are vital to immature birds during the first 2 or 3 weeks of life, and make up a much larger proportion of their diet during this time than at other times in their life. Thus, they are functionally insectivorous birds at this age. Also, for the purposes of pathogenicity testing, the Agency feels that sufficient immunological and physiological differences exist between immature birds and adult birds to warrant considering the immature bird as potentially more susceptible to infective challenge and so proposes their use in the maximum hazard testing approach.

(5) Selection of dose levels. For Tier I tests, the Agency suggests that a maximum hazard dosage be administered. For all testing, the maximum dose should be no less than the maximum hazard dose as defined in the testing guidelines (154A-1(h)1 and 154A-16 to 154A-24). If the MPCA produces significant toxic or pathogenic effects at the maximum hazard dose level, then testing at lower doses would be indicated. Sufficient doses and test organisms would be required to determine an LD_{50} value, if possible.

(6) Protocols. Interim protocols for some ecological effects testing have been developed by EPA's Office of Research and Development. Although these protocols have not been validated, they are available on request from EPA in order to provide guidance for applicants and testing laboratories in developing protocols for testing microbial pesticides on nontarget organisms.

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154A-3 Aquatic Animals.

(a) Approach. The Agency has considered several criteria that could be used to determine the extent of testing for effects on aquatic animals in Tier I. These are: the site of application and resulting potential for aquatic exposure; the natural geographic distribution of the microorganism; the natural population level of the microorganism compared with population levels likely after application; and, the ability of the MPCA to survive and replicate after application.

While all of these criteria are important, the Agency has chosen site of application and its resulting potential for aquatic exposure as the key criterion for establishing the extent of initial effects testing for MPCAs. The rationale for selecting this single criterion is that it directly addresses the most critical issue regarding potential hazard: likelihood of exposure. Furthermore, the other criteria would be implicitly considered in connection with the criterion for site of application.

The Agency recognizes that considerable judgment will be required to properly employ site of application as a criterion. While many uses obviously entail direct application to water (e.g., mosquito control and aquatic weed control), the Agency also intends that less obvious or borderline uses will be considered aquatic uses. Some examples that fall into the latter category are applications to forests, drainage ditches, riverbanks, and partially aquatic crops such as rice. Widespread applications to major crops such as cotton, soybeans, and corn could also warrant expanded testing if these crops are grown near bodies of water. To the extent possible, the Agency will rely on its experience with the classical chemical pesticides in distinguishing between terrestrial and aquatic use patterns in borderline situations.

(b) Tier Progression.

(1) Tier I. For MPCAs applied in terrestrial use patterns (where direct aquatic exposure is not anticipated), one freshwater fish (154A-19) and one freshwater aquatic invertebrate (154A-20) should be tested to assess toxicity and pathogenicity. For MPCAs applied directly to fresh, estuarine, or marine waters, one additional fish species and one additional invertebrate species should be tested in Tier I. These tests should be conducted as 30-day static renewal bioassays using one or a combination of methods to administer the pesticide (e.g., aqueous or dietary) These tests should be designed to simultaneously assess both toxicity and pathogenicity as well as to detect and quantify the microbial agent in the test animal. The concentration of MPCA in the water or food must be monitored to insure that the test organisms are exposed to a sufficient MPCA level throughout the test period.

No further testing would be indicated if: (1) results of the Tier I tests indicate no toxic or pathogenic effects, and (2) host range testing indicates that the MPCA has a narrow host range such that crossover into nontarget aquatic invertebrates is unlikely. If toxic or pathogenic effects are observed, then environmental expression testing (Tier II) would generally be required. In some cases, a subchronic test may serve to better understand the effects observed at the Tier I level and might alleviate the need for Tier II testing.

If host range testing implies crossover into nontarget aquatic invertebrates, then additional aquatic invertebrate species (those expected to be susceptible or likely to be exposed) would have to be tested in Tier I, or as an alternative, Tier II testing would have to be conducted. If tests on these additional species indicate toxic or pathogenic effects, then testing at Tier II would be indicated; if otherwise, then no further testing would be necessary.

(2) Tier II. The data for Tier II are described in environmental expression testing sections (155A) of these guidelines. If

the environmental expression characteristics do not indicate exposure of the MPCA to nontarget fish or aquatic invertebrates, then no further testing of these animals would be indicated. If Tier II tests indicate that fish and aquatic invertebrates will be exposed to the MPCA, then testing at Tier III is indicated.

(3) Tier III. Whereas Tier I tests are designed to screen MPCAs using a maximum hazard testing scheme, Tier III tests are intended to more precisely evaluate and quantify the actual hazard associated with the MPCA. The types of effects reported in Tier I tests would help determine which Tier III test(s) would be required. If only toxic effects are observed in Tier I tests, then the guidelines of 72-1 through -6 of Subdivision E would apply, and further testing would proceed as in Subdivision E. If pathogenic effects or both pathogenic and toxic effects are observed in Tier I, then tests that could be indicated in Tier III are the following: (1) Additional acute or subacute test(s) of fish or aquatic invertebrates to evaluate the spectrum of susceptible nontarget species, or determine the susceptible route(s) of exposure, or determine the dose-response relationship between the pesticidal agent and susceptible nontarget organism; (2) Aquatic invertebrate range testing (158A-27) and fish life cycle testing (154A-28); and/or (3) Aquatic ecosystem disruption studies (154A-29);

If results of Tier III tests indicate no pathogenic effects, then no further testing would be indicated. Conversely, if results of Tier III tests, along with environmental fate data, indicate toxic or pathogenic effects, then simulated or actual field testing (Tier IV) may be warranted.

(4) Tier IV. Simulated or actual field testing (154A-34) provides data on the pathogenic effects of the MPCA on fish and other aquatic animals following field applications at actual use rates. This test would apply when pathogenic effects are reported in Tier III testing (154A-27 and -29) at levels equal to actual or expected field exposure levels, and when the Agency is reasonably confident that quarantine methods can confine the MPCA to the test area and prevent contamination of adjacent areas. The specific test requirements would be determined on a case-by-case basis after consultation between the Agency and the registration applicant.

(c) Major issues. This section identifies and discusses issues regarding aquatic testing of MPCAs that may require further research and development. Most of the issues stem from two problems: (1) There are no standard widely accepted test protocols available to evaluate the effects of MPCAs on nontarget aquatic animals; and (2) There are some potential hazards associated with the use of MPCAs that the Agency recognizes and for which practical methods of evaluation are unavailable. The role of *in vitro* testing and Tier IV testing is also discussed in this section.

(1) Issues associated with Tier I protocol. Useful Tier I test protocols would simultaneously assess toxicity and pathogenicity in aquatic animals. The maximum hazard test philosophy would be exerted in terms of treatment level, method of pesticide administration, and age of the test animal.

A Tier I test should be conducted as a static renewal bioassay. The microorganisms should be administered: (1) as a suspension in the water (aqueous exposure); (2) in the diet in the form of diseased host insects or treated feed, or; (3) as a combination of both routes of exposure.

If any test animals die during the test, the cause of death (e.g., toxicity, pathogenicity) should be determined, if possible, and reisolation of the microorganism from test organism tissues should be attempted. This information would be used to determine what further tests, if any, are warranted. Exposure and observation should extend for at least 30 days for fish and 21 days for aquatic invertebates. Individual test animals should be removed periodically, if necessary, throughout the test period and at test termination for examination to assess pathogenicity.

If a sublethal infection is observed in test animals prior to test termination, it may be necessary to continue the observation period in order to more adequately assess the significance of the infection (e.g., will it be lethal?). Several published studies address certain aspects of the above-described protocol: Committee on Methods for Toxicity Tests with Aquatic Animals 1975; Ignoffo et al. 1973; Van Essen and Anthony 1976; Wolf 1975; Lightner et al. 13 Couch et al. 1975; and Hetrick, et al. 1979.

The following paragraphs discuss, in more detail, some of the Tier I aquatic organism tests.

(i) Test organisms. The guidelines provide that the species tested be selected from the list of species recommended by the Committee on Methods for Toxicity Tests with Aquatic Organisms (page 21) (1975), with the exception of goldfish (warmwater species; bluegill sunfish, channel catfish, and fathead minnow: coldwater species; rainbow trout, brook trout, coho salmon). These species are desirable test organisms for several important reasons: (1) They are used to evaluate chemical pesticides, and therefore EPA has considerable background data on these species; (2) Standard methods for the care and handling of these species are available; and (3) The species are widely distributed, are generally available, and have a variety of food habits and habitat requirements.

When possible, consideration should be given to testing species representative of the geographic region or ecosystem where the MPCA is to be applied. When applicable, species likely to prey upon or scavenge the diseased target host animals should be tested.

Unless there are other overriding considerations, the rainbow trout should be used as the freshwater fish test species. It is a desirable test animal because: (1) it is partially insectivorous; (2) no one species has been shown to be preferable in terms of sensitivity to MPCAs; (3) there is considerable background data on this species pertaining to its microbial diseases (Mann 1978); and (4) standard tissue culture procedures are available for this species (Wolf and Quimby 1969 and 1973).

Use of young fish (3 to 6 months old) is preferable since they would be more likely to display a lethal pathogenic effect, whereas older fish may become carriers.

Due to the broad phylogenetic spectrum from which to choose, it is difficult to select the most appropriate aquatic invertebrate. Generally, a test organism that is phylogenetically closest to the target host should be chosen. Such a test organism would be the most likely to be susceptible to infection by the MPCA. Therefore, when evaluating a MPCA whose target host is an insect, it would be appropriate to choose an aquatic insect (e.g., caddisfly) as the nontarget aquatic invertebrate test species.

Daphnia, a Cladoceran, has the advantage of having considerable background data for comparative purposes. Pound (1977) exposed the entomopathogen Mattesia to Daphnia and observed a bioconcentration effect. This resulted from the filter feeding habits of Daphnia and is a desirable feature in terms of assuring that the test animal ingests the microorganism. Both Daphnia and certain other aquatic insects have the advantage of a short life cycle or aquatic phase, and both undergo periods of natural stress and potential susceptibility to the microorganism as a consequence of molting.

(ii) Method of MPCA administration. Two methods of pesticide administration should be considered: (1) Suspension in the test water (aqueous exposure); and/or (2) Dietary, in the form of diseased target host animals or incorporation of the MPCA into a standard feed.

When possible, both routes should be used simultaneously in a single test to ensure that the most appropriate route of exposure has been tested and to insure a maximum challenge. Different pathogens may be capable of infection by different routes of exposure so that no single route may adequately screen all microorganisms. Each of the proposed routes has certain advantages and disadvantages. Therefore, a multiple route of exposure would be extremely beneficial and cost effective in screening MPCAs.

Addition of the microorganism directly to the test water is a routine procedure. It simulates the type of natural exposure that could occur immediately after application of a MPCA. It also simulates the route of exposure by which many known pathogenic agents infect fish and aquatic invertebrates. However, care must be taken to assure that a high concentration of microorganisms be maintained in the test system and that this high concentration does not lower water quality to an unacceptable level. Therefore, the static revewal method is recommended. Use of this method will insure that high MPCA concentrations and acceptable water quality can be maintained.

Dietary exposure also simulates certain natural conditions. In fact, it is perhaps the most important means of infection for the normal hosts of entomopathogenic agents (Surtees 1971). Therefore, its use in evaluating effects on nontarget fish and aquatic invertebrates is logical. This route offers a further advantage: It increases the possibility of exposing the test animals to a different life stage of the microorganism than may be present in the formulated product if diseased target hosts (e.g., insects) are used as the feed.

Finally, oral intubation of fish is another possible route of exposure, and is one that has been used to evaluate microorganism effects in fish (Savan et al. 1979; Narayanan et al. 1977). This route has the advantage of assuring that a known amount of test material is ingested. This advantage, however, does not outweigh the risk of injury or undue stress that could result from using this method. Therefore, the oral intubation method though acceptable is not recommended.

(iii) Test substance. The substance to be tested will depend in part on the method of pesticide administration used in the study. It is essential to test the most challenging form of the microorganism (in terms of pathogenicity or toxicity). It is equally important to test the form of the microorganism to which nontarget aquatic animals are most likely to be exposed. These objectives should be achievable through the use of multiple routes of administration, provided it is known which form is most challenging and which form is most likely to be encountered by the nontarget animal. The technical grade of the active ingredient should be used for all exposures. The formulated product should be tested if it is to be applied directly to water.

(iv) Selection of treatment concentrations. Treatment concentrations must be related to the number of microorganisms to which aquatic animals may be exposed under actual use conditions. And, in keeping with the maximum hazard philosophy, treatment concentrations must be relatively high. Consideration must be given to the level of exposure resulting from direct application as well as exposure resulting from consumption of diseased target host organisms (usually insects). Exposure in terms of frequency and number of microorganisms could be extremely high in the latter case.

The highest feasible concentrations should be used in all exposures. At a minimum, the concentration for aqueous exposure $1X10^6$ units/ml water or 1000 times the theoretical concentration present in 6 inches of water immediately after a direct application of the MPCA, at label rates, to 6 inches of water, whichever is greater and attainable. However, the use of such a high concentration may be limited by its adverse effect on water quality such as oxygen depletion and production of metabolic wastes by the microorganisms. Therefore, treated water in the test vessels should be renewed frequently enough to maintain water quality and microorganism concentration.

(v) Test duration. Exposure and observation must be extended to at least 30 days (unless test animals die) to allow time for any potential infection, microorganism replication, or pathogenic or toxic effects to manifest themselves. If a sublethal infection is observed, then the test should be extended to evaluate the significance of the infection. Similarly, if test animals begin to die near the end of the 30-day period, the test should be continued to determine the fate of the remaining test population.

The 30-day test duration was selected on the basis of past research [Savan et al. (1979); Pound (1977); Van Essen and Anthony (1976); and Lightner et al. (1973)]; and the recommendation of Summers et al. (1975). Certain factors may dictate that this period be modified. For example, if infection and death of target hosts is normally not evident for many days (i.e., 20 to 30), it would be logical to lengthen the period of exposure for the test animals. Conversely, a shorter period of exposure may be warranted in tests using animals with short life cycles (i.e., *Daphnia* or mysid shrimp).

(vi) Observation and examination of test animals. Daily observations are required to record mortalities and note any behavioral, pathogenic, or toxic effects. Test organisms must be examined for infection or any microorganism-related effects periodically throughout the study and at test termination. The most difficult aspect of this requirement is the verification of the presence or absence of an infection. The general methods of assessment that may be required to make this determination include histopathology, serology, and nucleic acid hybridization and reisolation and identification of the microorganism from organ tissue. These methods, and the situations in which their use may be appropriate, were presented in the general discussion of nontarget organism hazard testing, 154A-1 (b) (4).

Undeen and Maddox (1973) used the following criteria in their work with Nosema algerae to distinguish between a true infection and microorganisms observed in the test animal. In a true infection: (1) Both vegetative forms and spores had to be present in the test animal; and (2) The number of spores recovered had to exceed the number injected by 100X. This type of approach may be useful for other microorganisms.

(2) Issues associated with Tier III test protocols. The aquatic invertebrate host embryo larvae, fish life cycle, and aquatic ecosystem tests in Tier III (154A-27 through -29) are similar to the protocols that are referenced for these types of tests in Subdivision E of the Guidelines (77-4 through -7). Generally accepted standard protocols for conducting these studies with MPCAs have not been developed. In fact, few, if any, such tests have ever been conducted with MPCAs. Therefore, at the outset, the Agency recognizes that new and different test designs and test parameters may be more appropriate than modified Subdivision E tests. Research and methods development are in progress and need to be completed in this area before the Agency can publish specific recommendations concerning protocols and tier progression.

(3) The role of in vitro testing. The Agency recognizes that there are in vitro tests available to assess the infectivity of certain microorganisms, one of which is tissue culture for viruses. Cell lines are established for several species of fish (Wolf 1975), and such a test might be a useful means of assessing infectivity in certain situations. However, the relationship between effects demonstrated by in vitro tests and effects likely to occur under in vivo situations is uncertain. For example, Ignoffo (1975) states that "Tissue, completely nonsusceptible in the intact organism, may support viral multiplication when explanted into a culture media." Therefore, the results obtained from tissue culture tests could be useless in accurately predicting environmental hazard. Another potential drawback of tissue culture studies is that, often, no host cell culture (e.g., insect cell culture) has been developed. Therefore, such a study would have no positive control group and the validity of a negative result would always be subject to some doubt.

The Agency has concluded that, at the present time, in vitro studies such as tissue culture cannot be substituted for the in vivo studies provided in Tier I. At the same time, the Agency recognizes the potential value of these studies for the following purposes: (1) As a relatively inexpensive and rapid means to screen for potential infectivity in a broad spectrum of species; and (2) As a test to support or check the results of in vivo tests.

Therefore, a provision for cell culture studies is included in Tier III of the testing scheme.

(4) Tier IV Testing. The Agency recognizes the possible shortcomings in using simulated or actual field tests (Tier IV) as the final test of the safety of a MPCA. If an agent has progressed through the tier system and requires a field test, it must have displayed significant adverse effects in some or all of the previously conducted laboratory tests. This fact might argue against the use of a field test, since such a test could release potentially hazardous microorganisms, with the potential to proliferate in the environment and pose widespread environmental risk, unless adequate quarantine measures could be taken. Therefore, before any Tier IV field test is to be undertaken, the applicant should discuss its plans with the Agency concerning potential hazards. If the Agency determines that a Tier IV field test would pose an unacceptable risk, then the MPCA would not likely be acceptable for registration.

On the other hand, the Agency also recognizes the potential value of Tier IV simulated or actual field tests as a further check on the safety of MPCAs that demonstrate no hazard in Tier I tests, or that demonstrate a hazard that could be adequately controlled by quarantine methods in the field. These tests could be conducted concurrently with full-scale efficacy testing, and the Agency would strongly encourage such tests. This would provide the opportunity to evaluate pesticidal effects (both direct and indirect) on a much broader spectrum of nontarget species, under more natural exposure conditions, than is possible in Tier I testing.

(5) Assessment of other potential hazards: opportunistic infections and latent viruses. Opportunistic infections in nontarget aquatic animals are recognized by the Agency to be a potential hazard. A similar concern is noted for latent viruses. Research indicates that aquatic animals may be rendered significantly more susceptible to microbial infection, (e.g., by viruses and bacteria) when stressed by such factors as Aroclor 1254 (Couch and Courtney 1977), copper (Hetrick et al. 1979), temperature, salinity, pesticides, and other pollutants (Snieszko 1974; Schwartz 1974). This increased susceptibility raises several important questions: (1) What is the likelihood of an opportunistic infection (from a microbial pest control agent) occurring in a nontarget aquatic animal? What is the significance of the effect of opportunistic infections on individuals and populations? (2) Will the proposed Tier I test adequately screen MPCAs for potential opportunistic effects? Or could a MPCA be noninfective in a Tier I test, but infect stressed nontarget animals? (3) Will a latent virus be detected by a Tier I test and, if so, how can its significance be assessed?

There is far too little background information and research on MPCAs to suggest an answer to the first question. However, the Agency believes that the potential for this type of problem should not be ignored. The Agency is confident that sublethal infections produced in Tier I tests can be detected if the proper methods of detection are employed. However, the potential for an apparently noninfective agent (in Tier I testing) to infect stressed animals is unknown. At present, the Agency is not aware of any practical, generally accepted, routine screening test that could be used in Tier I to determine the potential for such an occurrence. If a sublethal infection is observed in Tier I, then further testing may be warranted. A microorganism/stress interaction test is proposed in Tier III as a means of assessing sublethal infections, but further research is needed to develop the protocol for such a test. With regard to latent viral infections, the Agency is not aware of a standard method to evaluate the potential for a latent virus to reactivate and cause adverse effects in aquatic animals. Further research is required.

(6) Oncogenic effects. The Agency recognizes the potential for oncogenic effects that are associated with viruses and mycotoxins. The probability of oncogenicity in nontarget aquatic animals, as a result of exposure to a viral pesticide, is unknown. At this time, the Agency is unaware of any standard method that could be used to screen for such an effect. Further research is required to develop an appropriate test and determine when its use is justified.

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154A-4 Nontarget Plant Testing.

(a) Approach. The plant testing scheme proposed herein is based on the tier testing scheme for testing other nontarget organisms. Tier I screening tests incorporates maximum hazard single dosing using a route of exposure most likely to show any potential plant toxicity or pathogenicity. The duration of the test should be sufficient to allow for manifestation of a delayed pathogenic response. Tier II testing examines population dynamics to quantify persistance and survival of the MPCA in the environment. In some cases, a subchronic test may serve to better understand the effects observed at the Tier I level and might alleviate the need for Tier II testing. Tier III testing is designed to record a dose response and determine if there is a minimum infective dose for any adverse effects identified in Tier I tests. Tier IV testing, if still needed for risk assessment, will include both exposure and hazard testing under simulated or actual field conditions.

(b) Discussion and major issues. Diseases of commercially important plants have been intensively studied for decades and many plant pathogens have been identified and subsequently well characterized. Some plant pathogens have a very narrow host range and may attack only one species of plant, whereas other plant pathogens may attack a wide range of plant species. Still other microorganisms have never been identified in association with disease in plants.

A thorough taxonomic description of the MPCA should allow determination of its similarity to known plant pathogens. MPCAs that are similar to plant pathogens with very narrow host ranges may only need to be tested for adverse effects against plants similar to known hosts. MPCAs that are similar to wide range plant pathogens may need additional testing to identify the complete host range. A knowledge of the mode of action may assist in determining the extent of testing needed for potential plant pathogens. Finally, MPCAs that do not resemble any known plant pathogen may require little, if any, plant testing. Microbial herbicides are designed to be toxic or pathogenic to their target plants. This class of MPCAs will require close scrutiny to ensure that nontarget plants are not unreasonably affected, whereas microbial insecticides generally would not be expected to have phytopathogenic properties, and would not require as much testing.

A second factor in determining the extent of plant testing is the anticipated exposure of plants to the MPCA as determined by the use pattern, dissemination, and the persistence /survival in the environment. For example, MPCAs that will not be disseminated to, or do not survive in, aquatic environments will not need testing in aquatic plants. A related factor is whether the MPCA is to be used within its area of natural occurrence. Where an MPCA is proposed for use in an area where it does not naturally occur, additional plant testing may be warranted.

Another factor in selecting species of plants to be tested is that of susceptibility to plant diseases. Genetically diverse groups of plants are generally less susceptible as a species to any given plant pathogen since there is a greater chance that a variety of the species will be resistant to the disease. The most important group of genetically identical (monoculture) plants are the commercial agricultural crops. These plants should be given priority in testing for plant pathogenicity because of both their potential susceptibility and their commercial importance.

154A-5 Nontarget Insects.

(a) Terrestrial insects.

(1) Approach. Assessment of potential nontarget insect hazard from uses of MPCAs is made difficult by a number of factors: (1) Most MPCAs will be specifically selected and/or designed for their ability to control pest insects. As such, nontarget insects represent the organism group most at risk, being, in most cases, relatively closely related to the target organism. (2) While there are few nontarget insects that have been shown to be economically important to humans, there are many nontarget insects which have an important role in ecological processes and may benefit humans indirectly. (3) Unlike chemical pesticides, many microbials will exert their effect through pathogenicity as well as toxicity. The acute, short duration, first tier tests, which should suffice for hazard evaluation for some chemical pesticides, will not be appropriate for microbial agents. Adequate assessment of pathogenicity will demand time to evaluate the MPCA for infectivity and for its ability to reproduce or develop in the test insect. (4) The host range is an important factor in hazard evaluation for a MPCA. A problem here is that extrapolation, even across species lines, is often not dependable. For this reason, the Agency will provide for testing with representatives from a number of "beneficial insect" taxa. Information from these tests will be used in conjunction with host range data (developed during efficacy testing) to develop a clearer idea of the overall insect host range.

The Agency is aware that this first tier of testing may, in some cases, be more extensive than the baseline data requirements in Subdivision L. However, there should be very few microbials which require effects testing beyond the Tier I level.

In view of the factors cited above, the tier-testing scheme for MPCAs is based on a fairly extensive first tier. The purpose of the Tier I testing is to assess toxicity and pathogenicity of the MPCA to the honey bee and to three species of predaceous and parasitic insects. Selection of the predator/parasite species to be tested should take into account such factors as the likelihood of exposure to the MPCA, phylogenetic proximity of the test species to target pest species, and similar relationships. A rationale for selection should be developed by the registrant.

Interim protocols for some nontarget insect effects testing have been developed by EPA's Office of Research and Development. Although these protocols have not been completely validated, they are available on request from EPA in order to provide guidance for applicants and testing laboratories in developing protocols for testing microbial pesticides on nontarget organisms.

(2) Tier Progression.

(i) Tier I. Under these guidelines, toxicity/pathogenicity tests on the honey bee and insect predators and/or parasites are indicated for all MPCAs. Selection of predator and parasite species for testing is made by the registration applicant. Species selected should be representative of groups which will be exposed under the conditions of proposed use, and which have some important relationship with the target pest. Rationale for selection is to be provided by the registrant. The main purpose of the Tier I testing is to determine presence of toxic or pathogenic effects on representatives of a few major orders of beneficial insects. As noted above, the representative test species selected, in addition to the honey bee, should be of some importance in the ecosystem to be exposed to the microbial control agent. Data derived from Tier I testing will be used in conjunction with available information on use pattern, host range (specificity), fate, and other similar factors, to assess potential for adverse effects. If data indicate no potential for adverse effects, no further testing would be indicated. Should the results of Tier I testing indicate toxic and/or pathogenic effects, then Tier II testing (environmental expression) would follow. In some cases, a subchronic test may serve to better understand the effects observed at the Tier I level and might alleviate the need for Tier II testing.

(ii) Tier II. The data for Tier II are described in environmental expression testing (155A) of these guidelines. If expression characteristics preclude exposure, no further testing would be indicated. If data indicate that nontarget insects will be exposed to the MPCA, then the registration applicant should consult with the Agency regarding possible Tier III testing.

(iii) Tier III. For all MPCAs, Tier III consists of advanced tests specifically responding to adverse effects identified in earlier tier testing. Such tests may be simulated or actual field tests, but further research is needed to develop the protocols for such testing. In any case, Tier III testing would be preceded by consultation with the Agency.

(b) Aquatic Insects. Tier I testing, as outlined in the "Aquatic Animal Tier Testing Scheme for Microbial Pest Control Agents" (154A-1) will include toxicity/pathogenicity testing with Daphnia, or a species of aquatic insect, or both, depending on use pattern. Detection of pathogenicity/toxicity in Tier I testing will automatically lead to expanded testing which, if the impacted site is fresh water, will most likely involve testing with aquatic insects.

TIER I TESTING

154A-16 Avian oral pathogenicity/toxicity test: Tier I.

(a) When required. Data on the oral pathogenicity of a MPCA to birds are required by 40 CFR 158.740(d) to support the registration of each end-use formulated product (EP) intended for outdoor application and each manufacturing-use product (MP) that legally may be used to formulate such an end-use product. See 40 CFR 158.50 and 158.740(d) to determine whether these data must be submitted.

(b) Test standards. Data must be derived from tests that satisfy the general test standards in 150A-3 of this subdivision and the following test standards:

(1) Test Substance. The actual form of the material to be regarded as the test substance is described in Section 154A-2(c)(2) of this document. In addition, any substances used to enhance virulence or toxicity should be tested along with the test substance.

(2) Species. Testing shall be performed on two avian species, one insectivorous and one herbivorous, (preferably bobwhite quail and mallard duck). The use of two species of birds with differing diets is recommended in order to take into account possible differences in gastrointestinal physiology. Other species may be used but a justification must be supplied based on increased susceptibility to

the Microbial Pest Control Agent (MPCA) or ecological considerations which preclude the use of recommended species.

(3) Age. Birds used in this test shall be from 14 to 24 days old at the beginning of the test period. Within a given test, all birds shall be as near the same age as possible.

(4) Controls.

(i) A negative, nondosed control group should be performed.

(ii) An infectivity control group should be performed and should be treated with MPCA inactivated in such a way as to retain the structural integrity of the cell.

(iii) A control group in which the birds are dosed with sterile filtrate from production cultures should be performed concurrently with the test groups.

(5) Number of birds per dosage level. Each treatment and control group shall contain at least 10 birds. When only one treatment group is tested, at least 30 birds shall be tested at that level.

(6) Maximum hazard dosage level. The highest oral dosage level tested is defined by the following formula:

$$\text{Max. daily dose (units)} = \text{MPCA Concentration in TGAI} \times \text{5 ml/kg Body Weight} \times \text{Weight of Test Bird (kg)}.$$

When using injection routes, substitute the following for "5 ml/kg Body Weight": intravenous, "0.5 ml/kg Body Weight"; and intraperitoneal, "2 ml/kg Body Weight".

For MPCAs that produce a toxin, fractions of this dose should be calculated for lower doses. This dose is then administered daily for 5 days. A justification shall be provided to support any reduction in the highest dosage level.

(7) Treatment conditions.

(i) If the MPCA produces a toxin, then a sufficient number of treatment doses must be tested to determine toxicity as described in paragraph (b)(9).

(ii) If the MPCA does not produce a toxin, or no toxin has been identified, then a single group of birds may be tested at the maximum hazard dose. If deleterious effects, due either to toxicity or pathogenicity, are observed, sequentially lower doses should be tested as described in paragraph (b)(9).

(8) Dosing regimen. Birds will receive oral doses daily for five days.

(9) Determination of an LD_{50} or ID_{50}. The study endpoint must be chosen to reflect the activity of the specific microorganism under test, *i.e.*, if an MPCA is expected to produce a toxin and has little or no infectivity, then the appropriate endpoint would be death of the test organism. If, however, the major mechanism is pathogenicity, then a more appropriate endpoint would be overt symptomatology.

The test data should establish that the avian oral LD_{50}, defined as the dose required to kill 50 percent of the test organisms, or ID_{50}, defined as the dose necessary to cause overt symptomatology in 50 percent of the test organisms, is greater than the maximum hazard dosage level. If the LD_{50} or ID_{50} is lower than the maximum hazard dose, then a definitive LD_{50} or ID_{50} with confidence limits should be established.

(10) Duration of test. Control and treated groups should be observed for at least 30 days after dosing initiation. If symptomatology or toxic signs are manifest at the thirtieth day, observation should continue until recovery, mortality or unequivocal moribundity is established.

(c) Reporting and evaluation of data. In addition to the information specified in 150A-4 of this subdivision, the test report shall contain the following information:

(1) Species;

(2) Age of the birds tested;

(3) Mean body weights for each test and control group at test initiation and weekly thereafter;

(4) Diet analysis (especially antibiotics);

(5) Pen dimensions;

(6) Ambient temperature and humidity;

(7) Photoperiod and lighting;

(8) Total feed consumption for each test and control group at weekly intervals;

(9) Method of test material preparation, concentration of the MPCA and total dose;

(10) Amount of test material dosed per bird;

(11) Amount of vehicle dosed per bird, if a vehicle other than water is used;

(12) Number of birds per group;

(13) ID50 or LD50 in appropriate units with 95% confidence limits if obtained;

(14) Methods used for calculation of LD50 or ID50;

(15) Slope of the dose response line, if obtained;

(16) Time and date of mortalities;

(17) Any signs of intoxication, abnormal behavior, and regurgitation (if any occurs);

(18) Reports of any pathogenic symptomatology or pathological changes;

(19) Results of gross necropsies and histopathological findings conducted on enough birds to characterize any gross lesions including attempts, using appropriate techniques, to reisolate the MPCA from examined tissues.

(d) Tier progression.

(1) If any pathogenic symptoms or toxic signs are observed at any dose level in this study, testing at Tier II, environmental expression testing (155A-15 through -23), is required as specified in 40 CFR 158.740. In some cases, a subchronic test may serve to better understand the effects observed at the Tier I level and might alleviate the need for Tier II testing.

(2) If toxic or pathogenic effects are not observed in this Tier I study, additional testing at higher tiers ordinarily is not required. The agency may require additional testing, however, if it determines that there is a potential risk to birds despite the negative Tier I results.

(e) References. The following references are provided for use in the development of acceptable test protocols for conducting an avian oral pathogenicity/toxicity test with a microbial pest control agent:

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154A-17 Avian respiratory pathogenicity test: Tier I.

(a) When required. Data on the avian acute inhalation pathogenicity of a MPCA are required by 40 CFR 158.740(d) to support the registration of each end-use formulated product (EP) intended for outdoor application and each manufacturinguse product (MP) that legally may be used to formulate such an end-use product. See 40 CFR 158.50 and 158.740(d) to determine whether these data must be submitted. The Agency recognizes that this test protocol has not yet been evaluated in the laboratory. An inhalation exposure may be acceptable in lieu of instillation and, for some microorganisms, the Agency may accept a request for waiver of this test with appropriate justification. It would be advisable to contact the Agency before performing the respiratory testing.

(b) Test standards. Data sufficient to satisfy the requirement in paragraph (a) of this section must be derived from tests which satisfy the purposes of the general test standards in 150A-3 of this subdivision, and all the following test standards:

(1) Test substance. The actual form of the material to be regarded as the test substance is described in Section 154A-2(c)(2) of this document. In addition, any substances used to enhance virulence or toxicity should be tested along with the test substance.

(2) Species. Testing shall be performed on one avian species (preferably bobwhite quail or mallard duck). Other species may be used but a justification must be supplied based on increased susceptibility to the MPCA or ecological considerations which preclude the use of recommended species.

(3) Age. Birds used in this test should be from 14 to 28 days old at the beginning of the testing period. Within a given test, all birds shall be as close to the same age as possible.

(4) Controls.

(i) A negative, non-dosed control group is required;

(ii) A concurrent control group is required and shall be treated with the pure active ingredient that has been inactivated in such a way as to preserve cellular integrity.

(iii) After dosing, two untreated contact control birds are required and shall be placed in with the treatment group receiving the maximum hazard dosage.

(5) Number of birds per dosage level. Each treatment and control group shall contain at least 10 birds. When there is only one treatment group at least 30 birds shall be tested at that treatment level.

(6) Route of exposure. The test material should be administered by intranasal or intratracheal instillation. Depending on the physical properties of the agent being tested, an alternate route, such as an aerosol, involving the respiratory tissues may be used. However, use of the alternate route must be justified.

(7) Dosing regimen. Birds should receive doses daily for five days.

(8) Maximum hazard dosage level. The maximum hazard dose is defined by the following formula.

$$\text{Max. daily dose (units)} = \text{MPCA Concentration in TGAI} \times \text{0.2 ml/kg Body Weight} \times \text{Weight of Test Bird (kg)}.$$

For MPCAs that produce a toxin, fractions of this dose should be calculated for lower doses. A reason shall be provided to support any reduction in the highest dosage level.

(9) Treatment concentrations.

(i) If the MPCA produces a toxin, then a sufficient number of treatment concentrations shall be tested to determine toxicity as described in paragraph (b)(10).

(ii) If the MPCA does not produce a toxin, or no toxin has been identified, then a single group of birds may be tested at the maximum hazard dose. If deleterious effects, due either to toxicity or pathogenicity, are observed, sequentially lower doses should be tested as described in paragraph (b)(10).

(10) Determination of an LD_{50} or ID_{50}. The study endpoint must be defined to reflect the pathological activity of the specific agent under test, i.e., if an MPCA is expected to produce a toxin and has little or no infectivity, then the appropriate endpoint would be death of the test organism. If, however, the major mechanism is pathogenicity, then a more appropriate endpoint would be overt symptomatology.

The test data must establish that the avian inhalation LC_{50}, defined as the dose required to kill 50 percent of the test organisms, or IC_{50}, defined as the dose necessary to produce overt symptomatology in 50 percent of the test organisms, is greater than the maximum hazard dosage level. If the LC_{50} or IC_{50} is less than the maximum hazard dose, then a sufficient number of treatment levels should be tested in order to obtain a definitive LC_{50} or IC_{50}, if possible.

(11) Duration of test. Control and treated birds should be observed for at least 30 days after dosing. If symptomatology is manifest at the thirtieth day, observation should continue until recovery, mortality or unequivocal moribundity is established.

(c) Reporting and evaluation of data. In addition to the information specified in 150A-4 of this subdivision, the test report shall contain the following information:

(1) Species;

(2) Age of the birds tested;

(3) Mean body weights for each test and control group at test initiation and weekly thereafter;

(4) Diet analysis (especially antibiotics);

(5) Pen dimensions;

(6) Ambient temperature and humidity;
(7) Photoperiod and lighting;

(8) Total feed consumption for each test and control group at weekly intervals;

(9) Method of test material preparation, concentration of the MPCA and total dose;

(10) Identification of vehicle or carrier used to disperse the agent and the average amount of vehicle administered to each bird, if a vehicle other than water is used;

(11) Number of birds per treatment level;

(12) Number of controls used;

(13) Time and date of mortalities or symptom onset;

(14) ID_{50} or LD_{50}, in appropriate units, with 95% confidence limits, if obtained;

(15) Results of gross necropsy and histopathological findings conducted on all birds dying before termination of the test, on a representative sample of those that survived, and on two contact control birds. The necropsy report should include any evidence of respiratory tract involvement and involvement at distant sites including liver, kidney, spleen, cerebrospinal system, gastrointestinal system. Blood samples should also be analyzed for abnormalities. Results should also include any attempts, using appropriate techniques, to reisolate the MPCA from examined tissues;

(16) A clinical assessment of any histopathological findings and lesions noted;

(17) Assessment of the clinical significance of MPCA tissue isolations.

(d) Tier progression.

(1) If any pathogenic or toxic effects are observed at the maximum hazard dosage level in this study, testing at Tier II [environmental expression testing (155A) is required as specified in 40 CFR 158.740(d). In some cases, a subchronic test may serve to better understand the effects observed at the Tier I level and might alleviate the need for Tier II testing.

134 (2) If no pathogenic effects are observed in this study, no additional testing at higher tiers ordinarily is required. The Agency may require additional testing, however, if it determines that there is a potential risk to birds despite the negative Tier I results.

(e) References. The following references are provided for use in the development of test protocols for conducting an avian inhalation pathogenicity test with microbial pest control agents:

(1) Friend, M. and D.O. Trainer 1970. Polychlorinated biphenyl: interaction with duck hepatitis virus. Science 170(3964):1314-1316.

(2) Friend, M. 1971. Experimental duck virus hepatitis in the mallard. Avian Disease 16(4):692-699.

(3) Friend, M. 1972. Duck hepatitis virus interaction with DDT and Dieldrin in adult mallards. Bull. Environ. Contam. Toxicol. 7(4):202-206.

(4) Friend, M. 1974a. Experimental DDT-Duck hepatitis virus interaction studies. J. Wildl. Manage. 38(4): 887-895

(5) Friend, M. 1974b. Experimental Dieldrin-Duck hepatitis virus interaction studies. J. Wildl. Manage. 38(4):896-904.

(6) Hartmann, G.C. and S.S. Wasti. 1980. Avian safety of three species of entomogenous fungi. Comp. Physiol. Ecol. 5(4): 242-245.

(7) Summers, M., R. Engler, L.A. Falcon, and P. Vail, eds. 1975. Pp. 179-184. in Guidelines for Safety Testing of Baculoviruses. Baculoviruses for Insect Pest Control: Safety Considerations. American Society for Microbiology Washington, D.C.

154A-18 Wild mammal toxicity and pathogenicity testing: Tier I.

(a) When required. Data on wild mammal toxicity and pathogenicity may be required by 40 CFR 158.740(d) on a case-by-case basis to support the registration of end-use formulated products (EP) intended for outdoor application and manufacturing-use products (MP) that legally may be used to formulate such end-use products. The toxicity and pathogenicity data required by the toxicology section of this subdivision for evaluating hazard to humans and domestic animals are normally adequate to indicate potential hazard to wild mammals. Under certain conditions, however, these data are not sufficient to assess the potential hazard to wild mammals likely to be exposed to a MPCA. An example of one circumstance when such additional testing be required is the situation in which data indicate that there is considerable variation in sensitivity of different mammalian species to the effects of a MPCA agent, and there is evidence that wild mammals will be heavily exposed to a MPCA. See 40 CFR 158.50 and 158.740(d) to determine whether these data must be submitted.

(b) Test standards. Data must be derived from tests that satisfy the general test standards in 150A-3 of this subdivision and the following test standards:

(1) Test substance. The actual form of the material to be regarded as the test substance is described in Section 150A-2(c)(2) of this document. In addition, any substances used to enhance virulence or toxicity should be tested along with the test substance.

(2) Species. Testing shall be performed on a mammalian species representative or indicative of those found in the area(s) likely to be affected by the proposed use pattern(s). Test animals may be reared in pens or captured in the wild, and must be phenotypically indistinguishable from wild mammals. Endangered or threatened animals shall not be used.

(3) Controls.

(i) A negative control group is required.

(ii) A concurrent control group is required and shall be treated, when possible, with the pure active ingredient that has been inactivated in such a way as to preserve cellular integrity.

(4) Route of exposure. The test material should be administered by gavage (acute oral dose) or by intranasal instillation. The method of dosing should reflect the expected exposure route and shall be determined after consultation with the Agency.

(5) Maximum hazard dosage level. The standards for maximum hazard dosage level, determination of an LD_{50} or ID_{50} and duration of test that are found in the avian oral pathogenicity/toxicity test 154A-16 and the avian inhalation pathogenicity test 154A-17.

(c) Reporting and evaluation of data. In addition to the information specified in 150A-4 of this subdivision, test reports shall contain the same information required for the avian oral pathogenicity/toxicity test 154A-16 and the avian inhalation pathogenicity test 154A-17, adapted appropriately for mammalian test procedures.

(d) Tier progression.

(1) If any toxic or pathogenic effects on mammalian species are observed at the maximum hazard dosage level in this study, testing at Tier II [environmental expression testing (155A) is required as specified in 40 CFR 158.740(d). In some cases, a subchronic test may serve to better understand the effects observed at the Tier I level and might alleviate the need for Tier II testing.

(2) If toxic or pathogenic effects are not observed in this study, additional testing at higher tiers ordinarily is not required. The Agency may require additional testing, however, if it determines that there is a potential risk to mammals despite negative Tier I results.

(e) *References*. The following references are provided for use in the development of test protocols for conducting wild mammal toxicity and pathogenicity tests with microbial pest control agents:

(1) Barnes, R.W., C.F. Meincke, W.C. McLane, and C.S. Rehnborg. 1970. Long-term feeding and other toxicitypathogenicity studies on rats using a commercial preparation of the nuclear-polyhedrosis virus of *Heliothis zea*. *J. Invert. Pathol.* 16:112-115.

(2) Fisher, R., and L. Rosner. 1959. Toxicology of the microbial insecticide, Thuricide. *Agricultural and Food Chemistry* 7(10):686-688.

(3) Ignoffo, C.M., and A.M. Heimpel. 1965. The nuclear polyhedrosis virus of *Heliothis zea* (Boddie) and *Heliothis virescens* (Fabricius) Part V. Toxicity-pathogenicity of virus to white mice and guinnea pigs. *J. Invert. Pathol.* 7:329-340.

(4) Ignoffo, C.M. 1971. Intraperitoneal injection of white mice with nucleopolyhedrosis virus of the beet armyworm, *Spodoptera exigua*. *J. Invert. Pathol.* 17(3):453-454.

(5) Ignoffo, C.M. 1973. Effects of entomopathogens on vertebrates. *Annals N.Y. Acad. Sci.* 217:141-164.

(6) Ignoffo, C.M., J.J. Petersen, H.C. Chapman, and J.F. Novotny. 1974. Lack of susceptibility of mice and rats to the mosquito nematode, *Reesimermis nielseni*, Tsai and Grundmann. *Mosquito News* 34(4):425-428.

(7) Ignoffo, C.M., C. Garcia, R.W. Kapp, and W.B. Coate. 1975. An evaluation of the risks to mammals of the use of an entomopathogenic fungus, *Nomuraea rileyi*, as a microbial insecticide. *In*: Baculoviruses for Insect Pest Control: Safety Considerations. Selected papers from EPA/USDA Working Symposium, Amer. Soc. Microb., Washington, D.C.

(8) Lamanna, C., and L. Jones. 1963. Lethality for mice of vegetative and spore forms of *Bacillus cereus* and *Bacillus cereus*-like insect pathogens injected intraperitoneally and subcutaneously. *J. Bacteriology* 85:532-535.

(9) Lautenschlager, R.A., C.H. Kircher, and J.D. Podgwaite. 1977. Effect of nucleopolyhedrosis virus on selected mammalian predators of the gypsy moth. USDA, For. Serv. Res. Paper, NE-377, 6p.

(10) Lautenschlager, R.A., and J.D. Podgwaite. 1979. Passage of nucleopolyhedrosis virus by avian and mammalian predators of the gypsy moth, *Lymantria dispar*. *Environ. Entomol*. 8(2):210-214.

(11) Lautenschlager, R.A., and J.D. Podgwaite. 1977. Passage of infectious nuclear-polyhedrosis virus through the alimentary tracts of two small mammal predators of the gypsy moth, *Lymantria dispar*. *Environ. Entomol.* 6(5):737-738.

(12) Meinacke, C.F., W.C. McLane, and C.S. Rehnborg. 1970. Toxicity-pathogenicity studies of a nuclear-polyhedrosis virus of *Heliothis zea* in white mice. *J. Invert. Pathol.* 15:10-14.

(13) Summers, M., R. Engler, L.A. Falcon, and P. Vail, eds. 1975. Pp.179-184 *in* Guidelines for Safety Testing of Baculoviruses Baculoviruses for Insect Pest Control: Safety Considerations. American Society for Microbiology Washington, D.C.

(14) Watts, D.M., R.F. Tammariello, J.M. Dalrymple, B.F. Eldridge, P.K. Russell, and F.H. Top, Jr. 1979. Experimental infection of vertebrates of the Pocomoke Cypress Swamp, Maryland with Keystone and Jamestown Canyon viruses. *Am. J. Trop. Med. Hyg.* 28(2):344-350.

(15) Wolf, K. 1975. Evaluation of the exposure of fish and wildlife to nuclear polyhedrosis and granulosis viruses. Pp. 109-111 *in* Baculoviruses for Insect Pest Control: Safety Considerations. American Society for Microbiology, Washington, D.C.

154A-19 Freshwater fish toxicity and pathogenicity testing: Tier I.

(a) When required. Data on pathogenicity and/or toxcity to fish are required by 40 CFR 158.740(d) to support the registration of each end-use formulated product (EP) intended for outdoor application and each manufacturing-use product (MP) that legally may be used to formulate such an end-use product. See 40 CFR 158.50 and 158.740(d) to determine whether these data must be submitted.

(b) Test standards. Data must be derived from tests that satisfy the general test standards in 150A-3 of this subdivision, and the following test standards:

(1) Test substance. The actual form of the material to be considered as the test substance is described in Section 154A-2(c)(2) of this document. In addition, any substance used to enhance virulence or toxicity should be tested along with the test substance.

(2) Test organisms.

(i) Testing shall be performed on one fish species, preferable the rainbow trout if the MPCA has only a terrestrial use and direct aquatic exposure is not expected or two fish species, preferably the bluegill sunfish and rainbow trout when direct aquatic exposure is anticipated. Other species of fish may be used, but a justification must be supplied based on an increased susceptibility to the MPCA or ecological considerations that preclude the use of recommended species.

(ii) The following characteristics should guide species selection:

(A) Fish species likely to prey upon or scavenge the target host organisms should be tested, when applicable.

(B) Testing of young fish is preferable. Very young (not yet actively feeding), spawning, or recently spent fish should not be used.

(C) Fish should weigh between 0.5 and 5.0 grams and be from the same year class. The length of the longest fish should be no more than twice that of the shortest fish.

(iii) Ten fish per group should be used in multiple group testing, thirty fish in single group testing.

(3) Route of Exposure.

(i) The test substance shall be administered as a suspension directly into the water (i.e., aqueous exposure).

(ii) Additionally, the MPCA should be administered through the oral route of exposure, preferably through incorporation in standard fish food or through the use of infected insects.

(4) Maximum Hazard Dose. At a minimum, the concentration in the test water (for aqueous exposure) should, whenever possible, be at least 10^6 units/ml of water or at least 1000 times the maximum calculated pesticide concentration in a six-inch layer of water immediately following a direct application to a six-inch layer of water, whichever is greater and attainable. Measures should be taken to insure that the initial concentration of the MPCA is main tained throughout the test should be described.

Feed used in the dietary exposure should be supplemented with the test substance to achieve a microbial concentration of at least 100 times the calculated cell density in a 6 inch layer of water immediately following a direct application to a 6 inch layer of water.

(5) Controls.

(i) A negative, nondosed control group should be run concurrently with the test groups.

(ii) A control group in which the fish are exposed to sterile filtrate from production cultures should be performed concurrently with the test groups.

(6) Test duration. The fish should be observed for a minimum of 30 days after dosing. If symptomatology is present at the thirtieth day, observation should be continued until recovery, mortality, or unequivocal moribundity is established.

(7) Treatment concentrations.

(i) If the MPCA produces a toxin, then a sufficient number of treatment concentrations must be tested to determine toxicity as described in paragraph (b)(8).

(ii) If the MPCA does not produce a toxin, or no toxin has been identified, then a single group of fish may be tested at the maximum hazard dose. If deleterious effects, due either to toxicity or pathogenicity are observed, sequentially lower doses should be tested as described in paragraph (b)(8).

(8) Determination of LC50 or ID50. The study endpoint must be chosen to reflect the activity of the specific microorganism under test, i.e. if an MPCA is expected to produce a toxin and has little or no infectivity, then the appropriate endpoint would be mortality. If, however, the major mechanism is pathogenicity, then a more appropriate endpoint would be overt symptomatology.

The data should establish that the freshwater fish LC_{50}, defined as the dose required to kill 50 percent of the test organisms, or IC_{50}, defined as the dose necessary to produce overt symptomatology in 50 percent of the test organisms is greater than the maximum hazard dosage level. If the LC_{50} or IC_{50} is lower than the maximum hazard dose then an LC_{50} or IC_{50} with confidence intervals should be established.

(c) Reporting and evaluation of data. In addition to information meeting the general reporting requirements of 150A-4 of this subdivision, a report of the results of a fish toxicity and infectivity test must include the following:

(1) LC_{50} or IC_{50} determination, including all associated parameters e.g. slope, goodness of fit etc., along with the raw mortality data.

(2) Detailed description of the steps taken to determine microorganism dissemination, replication, or survival in any test animals tissues, organs, or fluids:

(3) Detailed description of dilution water, including:

(i) Sterilization method;

(ii) Source;

(iii) Chemical characteristics (e.g., dissolved oxygen content, pH, chlorine content, dissolved salts); and

(iv) Pretreatments (if any).

(4) Detailed description of the test, including:

(i) Design;

(ii) Container size;

(iii) Medium (e.g., depth and volume);

(iv) Prophylactic treatments;

(v) Number of organisms per treatment level;

(vi) Loading (weight of organisms per unit volume of medium);

(vii) Lighting, acclimation, and test temperatures (averages and range);

(viii) Amount of test substance administered by each route of exposure; and

(ix) Any unusual feature of the test method.

(5) Detailed descriptions of methods (or references to established methods) used for all chemical analyses of water for chemical content and MPCA concentrations;

(6) Detailed description of methods used for all microbial analyses of water, transport hosts and test organisms, and results of such analyses.

(7) Detailed description of the effects of exposure to the test substance including:

(i) The criteria used to determine the effects;

(ii) Percentages of test animals that died or showed symptomology; and

(iii) A summary of these observations.

(8) Any additional relevant information about the test or its results that would assisst in the determination of hazard potential.

(d) Tier progression.

(1) If toxic or pathogenic effects are observed, then testing

at Tier II [environmental expression testing (155A) is required as specified in 40 CFR 158.740(d). In some cases, a subchronic test may serve to better understand the effects observed at the Tier I level and might alleviate the need for Tier II testing.

(2) Further testing generally is not required if results of this study do not indicate toxic or pathogenic effects. The Agency may require additional testing if it determines that there is a potential risk to fish despite negative Tier I results.

(e) References. The following may contain useful background information for developing test protocols:

(1) Anonymous. 1975. Standard Methods for Examination of Water and Wastewater. 14th Ed. American Public Health Assoc., Washington, D.C. 1193 pp.

(2) ASTM Standard E 729-80, Practice for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians. American Society for Testing and Materials, 1916 Race Street, Philadelphia, PA 19103.

(3) Committee on Methods for Toxicity Tests with Aquatic Organisms. 1975. Methods for acute toxicity tests with fish, macroinvertebrates, and amphibians. U.S. Environmental Protection Agency, Ecol. Res. Series, EPA 660/3-75-009. 61 pp.

(4) Hetrick, F.M., M.D. Knittel, and J.L. Fryer. 1979. Increased susceptibility of rainbow trout to infectious hematopoietic necrosis virus after exposure to copper. Appl. and Envir. Microb. 37(2):198-201.

(5) Huang, E., and J.S. Pagano. 1977. Nucleic acid hybridization technology and detection of proviral genomes. Chapter 13 in The Atlas of Insect and Plant Viruses, K. Maramorosch, Ed. Academic Press, New York.

(6) Ignoffo, C.M. et al. 1973. Susceptibility of aquatic vertebrates and invertebrates to the infective stage of the mosquito nematode *Reesimermis nielseni*. Mosquito News 33(4):599-602.

(7) Tatner, M.F., C.M. Johnson, and M.J. Horne. 1984. The tissue localization of *Aeromonas salmonicida* in rainbow trout, *Salmo gairdneri* Richardson, following three methods of administration. J. Fish Biol. 25: 95-108.

154A-20 Freshwater aquatic invertebrate toxicity and pathogenicity testing: Tier I.

(a) When required. Data on pathogenicity and/or toxicity to freshwater aquatic invertebrates are required by 40 CFR 158.740 to support the registration of each end-use formulated product (EP) intended for outdoor application and each manufacturing-use product (MP) that legally may be used to formulate such an end-use product. See 40 CFR 158.50 and 158.740(d) to determine whether these data must be submitted.

(b) Test standards. Data must be derived from tests that satisfy the general test standards in 150A-3 of this subdivision and the following test standards:

(1) Test substance. The actual form of the material to be regarded as the test substance is described in Section 154A-2(c)(2) of this document. In addition, any substances used to enhance the virulence or toxicity of the MPCA should be tested along with the test substance.

(2) Test organisms.

(i) For MPCAs having terrestrial use patterns, where direct aquatic exposure is not expected, one species of benthic invertebrate should be tested. For MPCAs where direct aquatic exposure is anticipated, testing shall be performed on two aquatic invertebrate species, one of which is planktonic and the other benthic.

(ii) The species of aquatic invertebrate selected should bear as close a taxonomic relationship to the target host as possible.

(iii) Aquatic invertebrate species likely to prey upon or scavenge the diseased target host organisms should be tested, when applicable.

(iv) Larval stages of invertebrates should be used whenever possible.

(v) Twent invertebrates/group should be used if there are multiple test groups. Fifty invertegrates should be used for single group testing.

(3) Controls.

(i) A negative, nondosed control group should be performed concurrently with the test groups.

(ii) A control group in which the invertebrates are exposed to sterile filtrate from production cultures should be performed concurrently with the test groups.

(4) Method of pesticide administration.

(i) The test substance shall be administered as a suspension directly into the water (i.e., aqueous exposure).

(5) Maximum hazard dose. At a minimum, the concentration in

the test water (for aqueous exposure) should, whenever possible, be at least 10^6 units per ml of water or at least 1000 times the maximum calculated pesticide concentration in a six-inch layer of water, immediately following a direct application to a six-inch layer of water, whichever is greater and attainable. The initial concentration of MPCA should be maintained throughout the test and the method used for maintainence of the dose level should be described.

(6) Test duration. The test duration should be at least 21 days. If pathogenicity and/or toxicity are apparent at the twenty-first day, observation should continue until recovery, mortality, or unequivocal moribundity is established.

(7) Treatment concentrations.

(i) If the test substance produces a toxin, then a sufficient number of treatment concentrations must be tested to permit a determination of toxicity as described in paragraphs (b)(8).

(ii) If the test substance does not produce a toxin, or no toxin has been identified, then a single group may be tested at the maximum hazard concentration. If deleterious effects, due either to toxicity or pathogenicity, are observed, then sequentially lower doses should be tested as described in paragraph (b)(8).

(8) Determination of EC50 or ID50.

(i) Satisfactory data must establish whether or not the test substance is pathogenic to the test organisms during a sufficiently long period of exposure and observation.

(ii) If the test substance produces a toxin, then the data must establish either:

(A) A definitive EC_{50} value with 95% confidence intervals; or

(B) That the EC_{50} is greater than the highest dose.

(c) Reporting and evaluation of data. In addition to information meeting the general reporting requirements of 150A-4 of this subdivision, a report of the results of an aquatic invertebrate toxicity and infectivity test must include:

(1) Raw data and EC_{50} calculations including 95% confidence intervals;

(2) A detailed description of the steps taken to determine microorganism dissemination, replication, or survival in the test animal tissues, organs, or fluids.

(3) A detailed description of dilution water, including source, chemical characteristics (e.g., dissolved oxygen content, pH, dissolved salts), method of sterilization, and pretreatment (if any).

(4) A detailed description of the test, including:

(i) Design;

(ii) Container size;

(iii) Medium (e.g., depth and volume);

(iv) Pretreatments, if any;

(v) Method of exposing organisms to the test substance;

(vi) Number of organisms per treatment;

(vii) Lighting, acclimation, and test temperatures (averages and range);

(viii) Amount of test substance administered; and

(ix) Any unusual feature of the test method.

(5) Detailed descriptions of methods (or references to established methods) used for chemical analyses of water for chemical content and MPCA concentrations.

(6) Detailed descriptions of methods used for all microbial analyses of water and test organisms, and the results of such analyses.

(7) Detailed description of the effects of exposure to the test substance, including:

(i) The criteria used to determine the effects;

(ii) Statement of percentages of organisms that died or showed effects of treatment; and

(iii) A summary of these observations, including changes in life cycle (duration, fecundity, and morphology).

(8) Any additional relevant information about the test or its results that would assist in the determination of hazard potential.

(d) Tier progression.

(1) If toxic or pathogenic effects are observed, then testing at Tier II [environmental expression (155A) shall be required. In some cases, a subchronic test may serve to better understand the effects observed at the Tier I level and might alleviate the need for Tier II testing.

(2) If no toxic or pathogenic effects are observed, then no

further testing at higher tiers ordinarily is required, except as noted in paragraph (d)(3) of this section.

(3) If host spectrum or beneficial insect tests indicate a broad host spectrum such that hazard toward aquatic invertebrates is indicated, then either:

(i) Additional aquatic invertebrate species must be tested as described in paragraphs (a) through (c) of this section; or

(ii) Testing at Tier II, environmental expression (155A) is required.

(4) If toxic or pathogenic effects are observed in tests conducted in accordance with paragraph (d)(3)(i) of this section, then testing at Tier II [environmental expression (155A) is required. If not, then no further tier testing is ordinarily required. The Agency may require additional testing, however, if it determines that there is a potential risk to aquatic invertebrates despite negative Tier I results.

(e) References. The following references may contain useful background information for developing test protocols:

(1) Anonymous. 1975. Standard Methods for Examination of Water and Wastewater. 14th Ed. American Public Health Assoc., Washington, D.C. 1193 pp.

(2) ASTM Standard E 729-80, Practice for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians. American Society for Testing and Materials, 1916 Race Street, Philadelphia, PA 19103.

(3) Committee on Methods for Toxicity Tests with Aquatic Organisms. Methods for Acute Toxicity Tests with Fish, Macroinvertebrates, and Amphibians. U.S. Environmental Protection Agency, Ecol. Res. Series, EPA 660/375-009. 61 pp.

(4) Huang, E., and J.S. Pagano. 1977. Nucleic acid hybridization technology and detection of proviral genomes. Chapter 13 in The Atlas of Insect and Plant Viruses, K. Maramorosch, ed. Academic Press, N.Y.

(5) Ignoffo, C. M. et al. 1973. Susceptibility of aquatic vertebrates and invertebrates to the infective stage of the mosquito nematode, Reesimermis nielseni. Mosquito News 33(4):599-602.

(6) Lightner, D.V., R.R. Proctor, A.K. Sparks, J.R. Adams, and A.M. Heimpel. 1973. Testing Penaeid shrimp for susceptibility to an insect Nuclear Polyhedrosis virus. Environ. Entomology 2(4):611-613.

(7) Pagano, J.S., and E. Huang. 1974. The application of RNA-DNA cytohybridization to viral diagnostics. *In*: Viral Immunodiagnosis. E. Kurstak and R. Morisset, eds. Academic Press, Inc. New York.

(8) Reynolds, G.J. 1978. Enzyme labelled antibody in histopathology. *Qualityline* (Winter 1978/1979):2-10.

(9) Summers, M., R. Engler, L.A. Falcon, and P. Vail, eds. 1975. Baculoviruses for Insect Pest Control: Safety Considerations. Selected papers from EPA-USDA Working Symposium, American Society for Microbiology Washington, D.C.

(10) Undeen, A.H., and J.V. Maddox, 1973. The infection of nonmosquito hosts by injection with spores of the microsporidan *Nosema algerae*. *J. Invert. Path*. 22:258-265.

(11) Van Essen, F.W., and D.W. Anthony. 1976. Susceptibility of nontarget organisms to *Nosema algerae* (Microsporida: Nosematidae), a parasite of mosquitoes. *J. Invert. Path*. 28:77-85.

(12) Weber, C.E. (ed.) 1973. Biological field laboratory methods for measuring the quality of surface waters and effluents. U.S. Environmental Protection Agency, Environ. Monit. Series, EPA-670/473-001.

154A-21 *Estuarine and marine animal toxicity and pathogenicity tests: Tier I*.

(a) *When required*. Data on pathogenicity and/or toxicity to estuarine and marine animals are required by 40 CFR 158.740(d) to support the registration of each end-use formulated product (EP) and of each manufacturing-use product (MP) that legally may be used to formulate such an end-use product for direct application into the estuarine or marine environment or expected to enter this environment in significant concentrations due to proposed use or mobility pattern. See 40 CFR 158.50 and 158.740(d) to determine whether these data must be submitted.

(b) *Test standards.* Data must be derived from tests that satisfy the general test standards in 150A-3 of this subdivision and the following test standards:

(1) *Test substance*. The actual form of the material to be regarded as the test material is described in Section 154A-2(c)(2) of this document. In addition, any substances used to enhance virulence or toxicity should be included.

(2) *Test organisms*.

(i) Toxicity and pathogenicity shall be determined for one species of shrimp, preferably Paleomonetes vulgaris and one estuarine or marine fish species.

(ii) Testing of additional estuarine or marine animal species may be required in Tier I as specified in paragraph (d)(3)(i) of this section.

(iii) Estuarine or marine animals likely to prey upon or scavenge the diseased target host organisms should be tested, when applicable.

(iv) Tests should be conducted using young fish, from the same year class, weighing between 0.5 and 5.0 grams. Very young (not yet actively feeding), spawning, or recently spawned fish should not be tested. The length of the largest fish should be no more than twice that of the shortest fish.

(3) Controls.

(i) A negative, nondosed control should be performed concurrently with the test groups.

(ii) A control group in which the animals are exposed to the sterile filtrate from the manufacturing-use product should be performed concurrently with the test groups.

(4) Method of pesticide administration.

(i) For fish, the MPCA wil be administered as a suspension directly into the water and, separately, incorporated into the food.

(ii) For shrimp, the MPCA will be incorporated into food pellets which are then fed to the test organisms.

(5) Maximum Hazard Dose. At a minimum, the concentration in the test water (for aqueous exposure) should, whenever possible, be at least 10^6 untis/ml or at least 1000 times the maximum calculated cell density in a six-inch layer of water immediately following a direct application to a six-inch layer of water, whichever is greater and attainable.

Feed used in the dietary exposure should be supplemented with the test substance to achieve a microbial concentration at least 100 times the calculated cell density in a 6-inch layer of water immediately following a direct application to a 6-inch layer of water.

(6) Test duration. The test should last at least thirty days. If pathogenicity and/or toxicity is apparent at the thirtieth day, observations should continue until recovery, mortality, or unequivocal moribundity is established.

(7) Number of organisms per concentration. The number of test organisms per group should be ten for fish and thirty for shrimp.

(8) Treatment concentrations.

(i) If the test substance produces a toxin, then a sufficient number of treatment concentrations must be tested to determine toxicity as described in paragraphs (b)(9)(ii) of this section.

(ii) If the test substance does not produce a toxin, or if no toxin has been identified, then a single group may may be tested at the maximum hazard dose. If deleterious effects, due either to toxicity or pathogenicity, are observed, sequentially lower doses should be tested as described in paragraph (b)(9).

(9) Determination of LC50 or ID50.

(i) Satisfactory data must establish an IC_{50} with 95% confidence limits or, that the IC_{50} is greater than the highest dose.

(ii) If the test substance produces a toxin, then the data must establish either:

(A) A precise LC_{50} or value with 95% confidence intervals; or

(B) That the LC_{50} is greater than the highest dose.

(c) Reporting and evaluation of data. In addition to information meeting the general requirements of 150A-4 of this subdivision, a report of the results of estuarine or marine animal toxicity and pathogenicity tests must include the following:

(1) LC_{50} data (if the test substance produces a toxin).

(2) A detailed description of the steps taken to determine microorganism dissemination, replication or survival in the test animal tissues, organs, or fluids;

(3) Detailed description of dilution water, including source, chemical characteristics (e.g., dissolved oxygen content, pH, chlorine content, dissolved salts), method of sterilization, and pretreatment (if any);

(4) Other pertinent details, including:

(i) Design;

(ii) Container size;

(iii) Medium (e.g., depth and volume);

(iv) Pretreatments, if any;

(v) Method of exposing organisms to the test substance (e.g., placing test substance in water which contains organisms or placing organisms in water which contains the test substance);

(vi) Number of organisms per treatment;

(vii) Loading (weight of organisms per unit volume of medium or unit of surface);

(viii) Lighting;

(ix) Acclimation and test temperatures (average and range);

(x) Salinities;

(xi) Amount of test substance administered;

(xii) Any unusual feature of the test;

(5) Detailed description of methods (or references to established methods) used for all chemical analyses of water for chemical content and MPCA concentrations;

(6) Detailed description of methods used in all microbial analyses of water and test organisms, and the results of such analyses.

(7) Detailed description of the effects of exposure to the test substance, including:

(i) The criteria used to determine the effects;

(ii) A statement of the percentage of organisms that died or showed effects from the treatment; and

(iii) A summary of these observations.

(8) Any additional relevant information about the test or its results that would assist in the determination of hazard potential.

(d) Tier progression.

(1) If toxic or pathogenic effects are observed, then testing at Tier II [environmental expression (155A) is required as specified in 40 CFR 158.740(d). In some cases, a subchronic test may serve to better understand the effects observed at the Tier I level and might alleviate the need for Tier II testing.

(2) If no toxic or pathogenic effects are observed, then no further testing at higher tiers is ordinarily required, except as noted in paragraph (d)(3) of this section.

(3) If efficacy or beneficial insect tests indicate a broad host spectrum such that susceptability of estuarine or marine invertebrates is indicated, then either:

(i) Additional estuarine or marine invertebrate species must be tested following the guidelines in paragraphs (a) through (c) of this section; or

(ii) Testing at Tier II [environmental expression (155A) is required as specified in 40 CFR 158.740(d).

(4) If toxic or pathogenic effects are observed in tests conducted in accordance with the requirements of this section, then testing at Tier II [environmental expression (155A) is required. Otherwise, no further tier testing is required.

(e) References. The following may contain useful background information for developing test protocols:

(1) Anonymous. 1975. Standard Methods for Examination of Water and Wastewater. 14th Ed. American Public Health Assoc., Washington, D.C. 1193 pp.

(2) ASTM Standard E 729-80, Practice for Conducting Static Acute Toxicity Tests with Larvae of Four Species of Bivalve Molluscs. American Society for Testing and Materials, 1916 Race Street, Philadelphia, PA 19103.

(3) Anonymous. 1978. Bioassay Procedures for the Ocean Disposal Permit Program. U.S. Environmental Protection Agency, Office of Research and Development. EPA-600/9-78-010. 121 pp.

(4) Bahner, L.H., C.D. Craft, and D.R. Nimmo. 1975. A salt-water flow-through bioassay method with controlled temperature and salinity. Prog. Fish-Cult. 37(3):126-129.

(5) Clark, J.R., and R. L. Clark, eds. 1964. Seawater systems for experimental aquariums. U.S. Int. Dept. Fish, and Wild. Serv., Bur. Sport. Fish. Wild. Res. Rep. No. 63, 192 pp.

(6) Committee on Methods for Toxicity Tests with Aquatic Organisms. 1975. Methods for acute toxicity tests with fish, macroinvertebrates, and amphibians. U.S. Environmental Protection Agency, Ecol. Res. Series, EPA 660/3-75-009. 61 pp. (Marine and estuarine species listed in this publication are acceptable.)

(7) Couch, J.A., M.D. Summers, and L. Courtney. 1975. Environmental significance of baculovirus infections in estuarine and marine shrimp. Annals N.Y. Acad. Sci. 219:528-536.

(8) Couch, J.A. 1984. Design and Test of a Simple System for the Preliminary Evaluation of Infectivity and Pathogenesis of Insect Virus in a Nontarget Estuarine Shrimp. J. Invert. Path. 43: 351-357.

(9) DeBen, E.A. 1970. Design and construction of saltwater environment simulator. Fed. Water Qual. Admin., Pacific N.W. Water Lab., Working Paper 71:1-30.

(10) Hetrick, F.M., M.D. Knittel and J.L. Fryer. 1979. Increased susceptibility of rainbow trout to infectious hematopoetic necrosis virus after exposure to copper. Appl. Environ. Micro. 37(2):198-201.

(11) Huang, E. and J.S. Pagano. 1977. Nucleic acid hybridization technology and detection of proviral genomes. Chapter 13 in The Atlas of Insect and Plant Viruses. K. Maramorosch, ed. Academic Press, N.Y.

(12) Ignoffo, C.M., et al. 1973. Susceptibility of aquatic vertebrates and invertebrates to the infective stage of the mosquito nematode, Reesimermis nielseni. Mosquito News 33(4):599-602.

(13) Lightner, D.V., R.R. Proctor, A.K. Sparks, J.R. Adams, and A.M. Heimpel. 1973. Testing Penaeid shrimp for susceptibility to an insect Nuclear Polyhedrosis virus. Environ. Entomology 2(4): 611-613.

(14) Pagano, J.S., and E. Huang, 1974. The application of RNA-DNA cytohybridization to viral diagnostics. In: Viral Immunodiagnosis. E. Kurstak and R. Morisset, eds., Academic Press, Inc., N.Y.

(15) Reynolds, G.J. 1978. Enzyme labelled antibody in histopathology. Qualityline (Winter 1978/1979):2-10.

(16) Shelbourne, H.E. 1962. Experimental seawater systems for rearing fish larvae. Pp.81-93 in Seawater Systems for Experimental Aquariums. J.R. Clark and R.L. Clark, eds. U.S. Dept. Int., Fish. Wild. Serv., Bur. Sport Fish. Wild. Res. Rep. No.63. 192 pp.

(17) Strickland, J.D.H., and T.R. Parsons. 1968. A practical handbook of seawater analysis. Fish Res. Board Can. Bull. No. 167., 311 pp.

(18) Summers, M., R. Engler, L.A. Falcon, and P. Vail, eds. 1975. Baculoviruses for Insect Pest Control: Safety Considerations. Selected papers from EPA-USDA Working Symposium, American Society for Microbiology Washington, D.C.

(19) Tatner, M.F., C.M. Johnson, and M.J. Horne. 1984. The tissue localization of Aeromonas salmonicida in rainbow trout, Salmo gairdneri Richardson, following three methods of administration. J. Fish Biol. 25: 95-108.

(20) Undeen, A.H., and J.V. Maddox. 1973. The infection of nonmosquito hosts by injection with spores of the microsporidan Nosema algerae. J. Invert. Path. 22:258-265.

(21) Van Essen, F.W., and D.W. Anthony. 1976. Susceptibility of nontarget organisms to Nosema algerae (Microsporida: Nosematidae), a parasite of Mosquitoes. J. Invert. Pathology 28:77-85.

(22) Weber, C.E. (ed.) 1973. Biological field laboratory methods for measuring the quality of surface waters and effluents. U.S. Environmental Protection Agency, Environ. Monit. Series, EPA-670/4-73-001.

154A-22 Plant studies: Tier I.

(a) When required.

(1) General. Data on the toxic or other adverse effects of a pesticide organism on plant growth and development are required by 40 CFR 158.740 to support the registration of each end-use product intended for outdoor application and each manufacturing-use product that legally may be used to formulate such an end-use product. See 40 CFR 158.50 and 158.740 to determine whether these data must be submitted.

(b) Test standards. In addition to satisfying the applicable general test standards outlined in 150A-3 of this subdivision, this study should meet the following standards:

(1) Test substance. The actual form of the material to be regarded as the test substance is discussed in Section 154A-2(c)(2) of this document. In addition, any substances used to enhance virulence should be tested along with the test substance.

(2) Dose levels. One concentration level equal to no less than the maximum label rate shall be tested. The phrase "the maximum label rate" means the amount of active ingredient in the recommended quantity of carrier, such as water to be used per land area or applied directly to the surface of a 15-cm or 6-in column of water.

(3) Test species. The number of species tested depends on the similarity of the MPCA to known plant pathogens. A rationale for selection of the species to be tested must be provided to the EPA.

(i) Animal-controlling MPCAs. When the pesticide is intended to control animals, including insects, the plants to be tested should include six species of Dicotyledoneae of at least four families and four species of Monocotyledoneae of at least 2 families. These species should be selected from the plants of most important commercial value (Table 3).

For MPCAs that have aquatic uses or may be expected to disseminate to, and survive in, aquatic ecosystems, additional aquatic plants must be tested to include: Selenastrum capricornutum (a freshwater green alga), Lemna gibba (duckweed), Skeletonema costatum (a marine diatom), Anabaena flos-aquae (a blue-green bacterium), and a freshwater diatom.

EPA will consider requests for waiver of part or all of this requirement if it can be shown that the MPCA occurs naturally in the area of intended usage and the level used does not exceed the naturally occurring concentration.

(ii) Plant-controlling MPCAs and MPCAs similar to known plant pathogens. When the pesticide is intended to control plant growth and development (microbial herbicides), or is otherwise closely related to a plant pathogen, in addition to testing the range of plants identified for animal-controlling MPCAs, testing must be performed on all plants of economic importance (horticultural/agronomic) or known to be beneficial to maintenance of the ecosystem that have any reasonable likelihood of serving as hosts. This selection of additional plant species should be based upon (1) a survey of plants closely related (same genus or, if not available, same family) to the target plant and (2) a survey of known hosts of pathogens closely related to the microbial herbicide.

(4) Controls. Both positive and negative controls should be included in the test protocols.

(i) Negative (untreated) controls should be as pest free as reasonably possible. In addition, in the case of MPCAs that are readily disseminated (wind, insects, etc.), it may be necessary to conduct tests such that negative controls and treated plants are grown in separate geographic locations or in separate contained greenhouses under identical environmental conditions so that reliable negative controls can be maintained. Alternatively, the negative control may be treated with a nonphytotoxic chemical pesticide known to provide effective control of the microbial pesticide. Since it is sometimes difficult to detect adverse effects, such as delayed maturation or loss in vigor, growth, quality, yield, or stand, it is important to analyze untreated controls using a sensitive, specific analytical method to determine whether or not MPCA infection has occurred.

(ii) Positive controls are required for microbial herbicides, or for MPCAs similar to known plant pathogens, in order to ascertain that environmental conditions are such that penetration, infection, and disease development are likely to occur in a susceptible host. The positve control should be selected to closely resemble the subject MPCA in terms of taxonomy and optimal conditions for infection and disease development, if known. In the case of a MPCA not intended for herbicidal use, the positive control may consist of a known plant pathogen, with taxonomic characteristics similar to the MPCA, and its susceptible host. In the case of a microbial herbicide, however, the positive control should consist of the target pest weed and the microbial herbicide.

(5) Environmental Test Conditions. When the optimum conditions for penetration, infection, and disease development are known or suspected, it is important, particularly for microbial herbicides, to simulate these conditions rather than those known to be optimum for plant growth and development. In many cases, however, the optimum environment may be similar.

(6) Application of MPCA. The test plants should be exposed to the MPCA by whatever route of exposure would be expected by the proposed use pattern. This route of exposure should be supplemented by other routes of exposure if indicated by the mode of transmission of typical pathogens of the test plant or, for microbial herbicides, if indicated by the mode of transmission of similar plant pathogens. In some cases, wounding of plants or simulation of (or actual) insect vectors might be appropriate. In other cases, seed treatment, root (soil) application, or foliar spray might be the most appropriate method.

(7) Timing of application. Plant test species should be treated at the time of most likely susceptibility (this may be known for microbial herbicides) or at the normal stage of maturity when application to target areas is initiated.

(8) Observations. Plants should be observed weekly or more frequently until normal harvest or death, or, in the case of perennials, at regular intervals for at least 2 years. If no obvious adverse effects are evident after these observation periods, the roots, foliage, fruit, vascular tissues, etc. should be analyzed for the presence of the organism using sensitive, specific methods. It is important to complete such analyses because (i) obvious disease development in perennials may take several years and (ii) asymptomatic plants may serve as sites for proliferation and survival of the organism, thus providing a reservoir of the organism in the environment.

(c) Reporting. In addition to the information specified in 150A-4 of this subdivision, the test report shall contain the following information.

(1) Rationale for selection of the species tested.

(2) Description of the growth chambers, greenhouse, or other type of test facility including containment provisions and monitoring devices for maintaining proper environmental conditions.

(3) Temperature and humidity ranges, including any significant deviations encountered in course of the experiment.

(4) Photoperiod and lighting.

(5) Any abnormal adverse or beneficial effects in treatment and/or control groups, including dates and times the effects were observed.

(6) Methods for any statistics used for analysis of results.

(d) Tier progression. If any adverse effects resulting from infection occur or analyses indicate that asymptomatic infection has occurred, testing at Tier II (Environmental Expression, 155A) is required as specified in 40 CFR 158.740.
In some cases, a subchronic test may serve to better understand the effects observed at the Tier I level and might alleviate the need for Tier II testing.

Table 3 - 25 Major Agricultural Crops

Listed by $ Value	Listed by lbs Produced
Corn	Corn
Soybeans	Alfalfa/Hay
Wheat	Wheat
Cotton	Soybeans
Tobacco	Sorghum
Sorghum	Sugarcane
Potatoes	Sugarbeets
Oranges	Potatoes
Barley	Oats
Rice	Oranges
Grapes	Grapes
Peanuts	Apples
Apples	Cotton
Sugarbeets	Grapefruit
Sugarcane	Peanuts
Tomatoes	Plums
Lettuce	Sunflower
Oats	Barley
Strawberries	Peaches
Beans	Tobacco
Onions	Beans
Peaches	Cucumbers
Almonds	Rice
Sunflower	Rye
Broccoli	Peas

NOTE: Depending upon the predicted use pattern, certain forest tree species, ornamental trees and shrubs, and weed species may need testing.

154A-23 Nontarget insect testing for toxicity/pathogenicity to insect predators and parasites: Tier I.

(a) When required. Data on the toxicity/pathogenicity of a microbial pest control agent are required by 40 CFR 158.740 to support the registration of each end-use product containing a microbial pest control agent intended for outdoor application when the proposed use pattern indicates that insect predators and/or parasites may be exposed to the pesticide, and each manufacturing use product that legally may be used to formulate such an end-use product. See 40 CFR 158.740 to determine whether these data must be submitted.

(b) Test standards. In addition to satisfying the applicable general test standards outlined in 150A-3 of this subdivision, this study should meet the following standards:

(1) Test substance. The actual form of the material to be regarded as the test substance is discussed in Section 154A-2(c)(2) of this document. In addition, any substances used to enhance virulence should be tested along with the test substance. Whenever feasible, dosage shall be in suitable increments up to 100X the LD_{50} or LC_{50} of the pathogen in its natural host, or 10 to 100 times the recommended field dosage.

(2) Test species. Testing should be performed on three species of insects, representing at least two of the following groups:

Parasitic dipterans
Predaceous hemipterans
Predaceous coleopterans
Predaceous mites
Predaceous neuropterans
Parasitic hymenopterans

Selection of test species is the responsiblity of the researcher. Rationale for selection must be provided. More specifically, the following points apply:

(i) Viruses.

Testing shall be performed on three species of insects representative of those parasites and predators known or suspected to attack the target host or to share the same ecological habitat.

(ii) Protozoa.

In selecting the 3 species of non-target arthropods, at least one should be a major (i.e., important) parasite of the target host. Many protozoans have a wide host range. Accordingly, if possible, more than the minimum (3) species of non-target organisms should be tested.

(iii) Fungi.
Assuming testing is justified, appropriate organism test species should be the major regulatory agents common to the ecosystem where the MPCA will be applied. In addition, testing could include parasites and/or predators specific to the host of the fungal agent.

(iv) Bacteria. Testing shall be performed on three species of insects representative of those parasites and predators known or suspected to attack the target host or to share the same ecological habitat.

(3) Controls. A concurrent control group is recommended and should be treated with microbe-free (or non-viable microbe) material from the culture system used for propagation of the microbial pest control agent.

(4) Routes of exposure.

(i) Viruses.

The best routes of exposure will depend on the developmental location of the nontarget organism. Internal stages may be tested with virus-infected hosts, or if they are culturable in vitro, the virus can be added to the diet. External stages, if they are obligatory, may be fed virus-infected hosts, and others may be fed virus-contaminated media or virus suspended in sugar or honey solutions.

(ii) Protozoa.

In addition to feeding adult predators and parasites of the target insect with the resistant stage of the protozoan, immature stages of the predator or parasite should be exposed. Predators can be fed hosts infected with the protozoan over a period of time. The predator, at a prescribed time, should be checked for protozoan infection.

The protocol for parasites is more complex. Protozoan-infected hosts can be parasitized or parasitized hosts can be fed protozoans. Parasites from protozoan-infected hosts should be examined for protozoan infection. The immature stages as well as adults should be examined. If possible, a primary hymenopterous and dipterous parasite should be examined.

The best route of infection for adult Hymenoptera or Diptera is oral acquisition of protozoan spores. Predaceous insects could also be fed in this manner, but feeding them infected (live) hosts of known age is more appropriate. In either case, the acutal amount of spores consumed cannot be accurately determined. If infection of the parasite or predator adult occurs, the possibility for transovarial or transovum transmission should be examined.

(iii) Fungi.
Routes of exposure should simulate field conditions as much as possible. In the case of entomopathogenic fungi, environmental conditions (>90% R.H.) are critical at the time of exposure. No scientific data is available on comparative exposure techniques.

(iv) Bacteria. Best routes of exposure will depend on developmental location of the nontarget organism. Internal stages may be tested with bacteria-infected hosts, or if they are culturable in vitro, the bacteria can be added to the diet. External stages may be fed bacteria-infected hosts, bacteria-contaminated media, or bacteria suspended in sugar or honey solutions.

(5) Duration of test/endpoints.

(i) Viruses.

Control and treated insects should be observed for a duration of at least 30 days after dosing, or in cases where an insect species cannot be cultured for 30 days, until control mortality rises above 20%. In cases where signs, symptoms and pathologies are detected, the treated insects should be examined in detail at late stages of infection, at moribund, and at death. Such tests need not be prolonged to 30 days if death of treated insects occurs prior to 30 days. In all cases of pathologies in the treated nontarget insects, it is essential that the etiology of the infection be established.

The end-points should be based on the frank development of pathologies and on the early mortality of the treated as compared to the untreated (control) nontarget organisms.

(ii) Protozoa.

Test duration should be determined on a case-by-case basis. The most appropriate end-point for protozoan diseases for determining pathogenic effects is the presence of the vegetative stages (shizonts or meronts) in the tissues of non-target insects. The schizonts within suspected tissues can be detected by making a smear, staining with Giemsa stain, and examining the slide with oil immersion using a compound microscope. The non-target insect should be alive when the tissues are removed for the smear because the shizonts are fragile and are usually destroyed by other microbes or are distorted upon death of the host.

Protozoan spores can be used as an indicator of infection. However, if the infection is light, the few spores could have come from the inoculum. If spores are abundant (a relative term) and occur in the tissues of the nontarget insect, it is likely that it is infected.

Another way to confirm infection is to conduct histological studies of the tissues using standard methods and looking for spores and other pathological effects. The end-point would be just before death of the organism or a prescribed period of time.

Death of the non-target insect is a good end-point if the protozoan is virulent. However, since most protozoans have chronic effects on their host, changes in behavior, size, or color could be used as an end-point. In each case, a microscopic examination to find schizonts or spores is essential to confirm the presence of the protozoan. Koch's postulates should be run.

(iii) Fungi.

With entomopathogenic fungi, test duration can be limited to 8-10 days.

Mortality in time LT(50) is considered the most reliable parameter for bioassaying fungi of insects in the laboratory. Pathogenicity should be confirmed by identifying the fungal agent as the original inoculum.

(iv) Bacteria.

Control and treated insects should be observed for 21 to 30 days after dosing, if this is possible. In cases where the insect species cannot be cultured for 21 to 30 days, observation should continue until control mortality rises above 20%.

(c) Reporting and evaluation of data. The reporting provisions are the same as those specified in 150-4 of this subdivision.

(d) Tier progression.

(1) Data derived from Tier I testing will be used in conjunction with available information on use pattern, host range, and other similar factors, to assess potential for adverse effects. If data indicate potential for adverse effects, Tier II testing will be required as specified in 40 CFR 158.740. In some cases, a subchronic test may serve to better understand the effects observed at the Tier I level and might alleviate the need for Tier II testing.

(2) If toxic or pathogenic effects are not observed in this study, additional testing is ordinarily not necessary.

(e) References.

The following references are provided for use in the development of acceptable test protocols for conducting toxicity/pathenogenicity tests with microbial pesticides:

Andreadis, T.G. 1980. Nosema pyrausta infection in Macrocentrus grandii, a braconid parasite of the European corn borer, Ostrinia nubilalis. J. Invertebr. Pathol. 35:229-233.

Brooks, W.M. 1973. Protozoa: Host - parasite - pathogen interrelationships. Misc. Pub. Entomol. Soc. Amer. 9:105-111.

Gardner, W.A., R.D. Oetting, and G.K. Storey. 1982. Susceptibility of the two-spotted spider mite, Tetranychus urticae Koch, to the fungal pathogen Hirsutella thompsonii Fisher. Florida Entomol. 65(4): 458-465.

Van Essen, F.W., and D.W. Anthony. 1976. Susceptibility of nontarget organisms to Nosema algerae (Microsporida: Nosematidae), a parasite of mosquitoes. J. Invert. Pathol. 28: 77-85.

154A-24 Honey bee toxicity/pathogenicity test: Tier I.

(a) When required. Data on the toxicity/pathogenicity of a microbial pest control agent are required by 40 CFR 158.740 to support the registration of each end-use product intended for outdoor application when the proposed use pattern indicates that honey bees may be exposed to the pesticide, and for each manufacturing-use product that legally may be used to formulate such an end-use product. See 40 CFR 158.50 and 158.740 to determine whether these data must be submitted.

(b) Test standards. In addition to satisfying the applicable general test standards outlined in 150A-3 of this subdivision, this study shall meet the following standards:

(1) Test substance. The actual form of the material to be regarded as the test substance is discussed in Section 154A-2(c)(2) of this document. In addition, any substances used to enhance virulence should be tested along with the test substance.

(2) Test species. Testing shall be performed on the honey bee, Apis mellifera.

(3) Age. Test insects should be worker bees of uniform age.

(4) Controls. A concurrent control group is recommended and should be treated with microbe-free (or non-viable microbe) material from the culture system used for propagation of the microbial pest control agent.

(5) Duration of test. Control and treated bees should be observed for at least 30 days after dosing.

(c) Reporting and evaluation of data. The reporting requirements are the same as those specified in 150A-4 of this subdivision.

(d) Tier progression.

(1) Data derived from Tier I testing will be used in conjunction with available information on use pattern, host range, and other factors, to assess potential for adverse effects. If data indicate that the potential for adverse effects exists, Tier II testing will be required as specified in 40 CFR 158.740. In some cases, a subchronic test may serve to better understand the effects observed at the Tier I level and might alleviate the need for Tier II testing.

(2) If toxic or pathogenic effects are not observed in this study, additional testing is ordinarily not necessary.

(e) References. The following references are provided for use in the development of acceptable test protocols for conducting a honey bee toxicity/pathogenicity test with a microbial pest control agent:

(1) Davidson, W.W., H.L. Morton, J.O. Moffett, and S. Singer. 1977. Effect of Bacillus sphaericus strain SSII-1 on honey bees, Apis mellifera. J. Invert. Pathol. 29:344-346.

(2) Menapace, D.M., R.R. Sackett, and W.T. Wilson. 1978. Adult honey bees are not susceptible to infection by Nosema locustae. J. Econ. Entomol. 71:304-306.

(3) Morton, H.L., J.O. Moffett, and F.D. Stewart. 1975. Effect of alfalfa looper nuclear polyhedrosis virus in honey bees. J. Invert. Pathol. 24:139-140.

(4) Hitchcock, J.D., A. Stoner, W.T. Wilson, and D.M. Menapace. 1979. Pathogenicity of Bacillus pulvifaciens to honey bee larvae of various ages (Hymenoptera: Apidae). J. Kansas Entomol. Soc. 52(2): 238-246.

TIER III TESTING.

154A-25 Terrestrial wildlife and aquatic organism toxicity testing: Tier III.

(a) When required. The data outlined in section series 71 and 72 of Subdivision E are required by 40 CFR 158.740 to support the registration of each end-use product and each manufacturing-use product that may be legally used to formulate such an end-use product when toxic effects on nontarget terrestrial wildlife or aquatic organisms are reported in one or more Tier I tests (154A-16 through -21) and results of Tier II tests (section series 155A) indicate exposure of the MPCA to the affected nontarget terrestrial wildlife or aquatic organisms. See 40 CFR 158.50 and 158.740 to determine whether these data must be submitted.

(b) Test standards. The test standards are the same as those found in 71-1 through -5 and 72-1 through -6 of Subdivision E.

(c) Reporting and evaluation of data. The reporting and evaluation provisions are the same as those found in 71-1 through -5 and 72-1 through -6 of Subdivision E.

(d) Tier progression. Further testing shall be required as specified in 40 CFR 158.740 and outlined in 71-1 through -5 and 72-1 through -6 of Subdivision E.

154A-26 Chronic avian pathogenicity and reproduction test: Tier III.

(a) When required. Data on the chronic avian pathogenicity and reproduction effects of a MPCA are required by 40 CFR 158.740(d) to support the registration of each end-use formulated product (EP) intended for outdoor application and each manufacturing-use product (MP) that legally may be used to formulate such an end-use product when:

(i) Pathogenic effects are observed in Tier I (154A-16 and -17) at any dose level and

(ii) Environmental expression testing (155A) indicates that long-term exposure of terrestrial animals to low-level field residues of the MPCA is likely. See 40 CFR 158.50 and 158.740(d) to determine whether these data must be submitted.

The test is intended to determine if, when animals are exposed to low levels of the MPCA over an extended period of time, chronic effects will occur.

(b) Test standards. Data must satisfy the general test standards in 150A-3 of this subdivision, and the following test standards:

(1) Test substance. The actual form of the material to be tested is described in Section 154A-2(c)(2) of this document. In addition, any substances used to enhance the virulence should be tested along with the test substance.

(2) Species. Testing shall be performed on one avian species (preferably the bobwhite quail or mallard duck). The species selected shall be the same as that used for the avian inhalation pathogenicity test in 154A-13.

(3) Age. Birds approaching their first breeding season shall be used.

(4) Controls. A concurrent control group is required and shall be treated with inactivated MPCA.

(5) Treatment levels. At least two treatment levels shall be used. The test concentrations should include an actual or expected field residue exposure level and a multiple of that level such as 5x.

(6) Dosing. The MPCA should be incorporated into the diet whenever possible. The diet should be analyzed for MPCA viability and new diet should be prepared often enough

so as to insure that the test animals receive a constant dose throughout the study. Alternate routes of dosing, along with the rationale, should be discussed with the Agency.

(7) Number of birds per treatment group. Each treatment group should be replicated. For bobwhite quail and mallard ducks, a minimum of 12 pen replicates for each treatment level should be used.

(8) Duration of exposure. Birds shall be exposed to treated diets beginning not less than 10 weeks before egg laying is expected, and extending throughout the laying season.

(9) Duration of test. The test shall not end before 14 days after the last hatchling leaves the shell.

(c) Reporting and evaluation of data. In addition to the information specified in 150A-4 of this subdivision, the test report shall contain the following information:

(1) Testing protocols. All methods used in performing these tests should be fully referenced or described in detail.

(2) Test results. The following information shall be reported for all test groups:

(i) Any observed abnormal behavior;

(ii) All results of tests used to detect the presence of the MPCA;

(iii) Time and date of mortalities;

(vii) Results of gross necropsy tests conducted on all birds dying or manifesting clinical symptomatology at test termination. Necropsies should also be performed on at least 50 percent of the nonaffected birds.

(viii) Reisolation of microbes from selected body tissues that would normally be affected by an infection including the liver, kidney, lungs, spleen, cerebrospinal system, testes ovaries, oviducts, and gastrointestinal tract and from all dead embryos, and an assessment of the clinical significance of such isolations;

(ix) Morbidity;

(x) Accidental deaths or injuries; and

(xi) Observed clinical signs and symptoms

(3) Test conditions. The following information shall be reported for treated and untreated test groups:

(i) Species;

(ii) Age;

(iii) Body weight;

(iv) Number and sex of birds in each treatment group;

(v) Individual identification of birds;

(vi) Diet composition and additives (especially antibiotics;

(vii) Diet storage conditions and results of periodic assays for MPCA content;

(viii) Feed consumption (grams per day);

(ix) Observation on palatability or repellency;

(x) Housing conditions of test birds:

(A) Space allocations for mating and nesting;

(B) Measures taken to insure that the birds were protected from injuries;

(C) Lighting program, including hours per day and wattage or footcandles at bird level;

(xii) Diagram of test layout:

(xiii) Temperature;

(xiv) Water source and its microbiological and chemical characteristics if sterilized distilled water is not used; and

(xv) Pretest and test history of the test organisms with respect to medical and chemical treatments.

(3) Egg and hatching data.
The following information shall be reported for each treatment

group:

(i) Eggs laid (number eggs per bird per day and per season);

(ii) Hatching egg storage data:

(A) Temperature;

(B) Humidity;

(C) Number of eggs placed in incubator;

(D) Egg-turning frequency;

(iii) Number of embryonated eggs;

(iv) Number of embryos that hatch;

(v) Ratio of hatched embryos to the number placed in the incubator;

(vi) Number of dead embryos;

(vii) Physical abnormalities in hatchlings;

(viii) All observed clinical signs and symptoms;

(ix) Post-hatchling mortality; and

(x) Weights of fourteen-day-old survivors.

(4) Pesticide test data. The concentration of the MPCA in the feed must be monitored weekly, more often if MPCA viability is short, and the results tabulated. For large quantities of treated feed, a statistically-sound sampling method should be employed.

(d) Tier progression.

(1) If pathogenic and/or reproduction effects are observed at actual or expected exposure levels:

(i) The applicant should reconsider the proposed registration of the product; and

(ii) The Agency will, at this time, review all the data and determine if a decision regarding acceptability for registration should be made. Testing at Tier IV, simulated or actual field testing (154A-33) may not be feasible if adequate containment or quarantine methods are not possible. If they are, testing at Tier IV is required as specified by 40 CFR 158.740(d).

(2) If no pathogenic effects are observed at actual or expected field residue exposure levels, no additional testing is ordinarily required.

(e) References. The following references are provided for use in the development of test protocols for conducting chronic avian pathogenicity and reproduction tests with microbial pest control agents:

(1) Heinz, G.H. 1976. Methylmercury: Second year feeding effects on mallard reproduction and duckling behavior. J. Wildl. Manag. 40(1):82-90.

(2) Heinz, G.H., and L.N. Locke. 1976. Brain lesions in mallard ducklings from parents fed methylmercury. Avian Diseases 20(1):9-17.

(3) U.S. Environmental Protection Agency, Registration of Pesticides in the United States: Proposed Guidelines, Subdivision E--Hazard Evaluation: Wildlife and Aquatic Organisms. 1978 (July 10). Fed. Reg. 43(132):29729-29731.

154A-27 Aquatic invertebrate range testing: Tier III.

(a) When required. Data from aquatic invertebrate range testing are required by 40 CFR 158.740(d) to support the registration of each end-use formulated product (EP) intended for use in water or expected to be transported to water from the intended use site, and when pathogenicity or infectivity was observed in Tier I tests on aquatic invertebrates. These tests are required to support the registration of each manufacturing-use product (MP) that legally may be used to formulate such an end-use product. See 40 CFR 158.50 and 158.740(d) to determine whether these data must be submitted.

(b) Test standards. Data must be derived from tests that satisfy the general test standards in 150A-3 of this subdivision, and the test standards in Tier I (154A).

(1) Test organisms. In selecting invertebrate species for this test, one should strive to choose species most likely to be affected by the MPCA under test. Thus, if a microorganism that is closely related (within the same family) to the MPCA causes disease in a certain invertebrate species, that species should be included in this test.

Use pattern also plays a role in selection. Microbial pest control agents that are expected to enter freshwater ecosystems should be tested on freshwater aquatic invertebrates. Likewise, if an estuary is likely to be impacted, marine invertebrates should be included.

In any case, testing should be performed on two members of the following orders.

Freshwater

Cladocera
Copepoda
Ephemeroptera
Trichoptera

Marine

Copepoda
Crustacea

(3) Method of pesticide administration. The test substance shall be administered either as a suspension in the test water (aqueous exposure) and/or in the diet as determined from results of Tier I tests.

(4) Dose levels. The dose level shall be equal to that expected to be found in the aquatic environment calculated from application rates with appropriate adjustments to take into account the environmental survival and multiplication characteristics, as determined by Tier II testing, of the MPCA under test.

(5) Test duration. The test duration shall be the same as Tier I studies.

(c) Reporting and evaluation of data. The requirements in Tier I, 154A-16 and -17, apply.

(d) Tier progression.

(i) If pathogenic effects are observed, further testing at Tier IV (154A-29) may be required.

(ii) If pathogenic effects are not observed, additional testing at higher tiers ordinarily is not required.

(e) References. Refer to paragraph (e) in 154A-20 and -21.

154A-28 Fish life cycle studies: Tier III.

(a) When required. Data from fish life cycle studies are required by 40 CFR 158.740(d) to support the registration of each end-use formulated product (EP) intended for use in water or expected to be transported to water from the intended use site, and when pathogenicity or infectivity was observed in Tier I tests. These tests are required to support the registration of each manufacturing-use product (MP) that may be legally used to formulate such an end-use product. See 40 CFR 158.50 and 158.740(d) to determine whether these data must be submitted.

(b) Test standards. Data must be derived from tests that satisfy the general test standards in 150A-3 of this subdivision, and the following test standards:

(1) Test substance. The actual form of the test material to be used is described in Section 154A-2(c)(2) of this document. In addition, any substances used to enhance virulence should be tested along with the test substance.

(2) Duration of test. The fish life-cycle tests require that the test animals be cultured in the presence of the MPCA during one complete life cycle, *i.e.* one stage of the life cycle to the same stage of the next generation.

(3) Test organisms. In selecting a species of fish for this test, one should strive to choose the species most likely to be affected by the MPCA under test. Thus, if a microorganism that is closely related (within the same family) to the MPCA causes disease in a certain fish species, that species should be chosen as the test organism.

Use pattern also plays a role in the selection. Microbial pest control agents that are expected to enter freshwater ecosystems should be tested on freshwater fish. Likewise, if an estuary is likely to be impacted, marine fish should be used.

In the event that a species cannot be identified using the foregoing criteria, suggested species are as follows.

(i) Freshwater species:

- Brook trout (Salvelinus fontinalis)
- Fathead minnow (Pimephales promelas)
- Bluegill sunfish (Lepomis marochirus)
- Channel catfish (Ictalurus punctatus)

(ii) Marine/Esturine species:

- Sheepshead minnow (Cyprinodon variegatus)
- Atlantic silverside (Menidia menidia)

(4) Dose levels. The dose levels shall be equal to that expected to be found in the aquatic environment cxalculated from application rates with appropriate adjustments to take into account the environmental survival and multiplication characteristics, as determined by Tier II testing, of the MPCA under test.

(5) Test procedure. Refer to the listed references for general guidance in design of fish life cycle tests. The applicant should consult with the Agency for testing details.

(6) Test duration. For test species with life cycles of a year or less, the test should begin with newly-hatched larvae and continue until thirty days after progeny hatch. When fish with life cycles that are greater than one year, begin the test with fish that are about to commence their first spawn and continue until ninety days after the progeny hatch.

(c) Reporting and evaluation of data. In addition to the information specified in 150A-4 of this subdivision, the test report shall contain the following information on the nontarget test organism:

(1) Reproductive effects;

(2) Effects on the growth of larvae and juveniles;

(3) Detailed records of spawning, egg numbers, fertility, and fecundity;

(4) Estimated no observed effect level;

(5) Mortality data;

(6) Statistical evaluation of effects;

(7) Locomotion, behavioral, physiological, and pathological effects;

(8) Definition of the criteria used to determine effects;

(9) Summary of observed signs of pathogenicity or other effects;

(10) Isolation, identification, and enumeration of microorganism(s) responsible for any observed pathogenic effects; and

(10) Stage of life cycle in which effects occurred.

(d) Tier progression.

(1) If pathogenic effects are observed, further testing at Tier IV 154A-33 may be required.

(2) If pathogenic effects are observed, additional testing at higher tiers ordinarily is required.

(e) References. The following may contain useful background information for developing acceptable protocols:

(1) Fish life-cycle tests:

(i) Anonymous. 1985. Standard Methods for Examination of Water and Wastewater. 16th Ed. American Public Health Assoc., Washington, D.C. 1268 pp.

(ii) Biesinger, K.E. 1974(a). Procedure for *Daphnia magna* tests in standing system. U.S. Environmental Protection Agency, Environ. Res. Lab., Duluth, Minn.

(iii) Biesinger, K.E. 1974(b). Procedure for Daphnia magna chronic tests in flowing system. U.S. Environmental Protection Agency, Environ. Res. Lab., Duluth, Minn.

(iv) Hansen, D.J., P.R. Parrish, S.C. Schimmel, and L.R. Goodman. 1978. Life-cycle toxicity test using sheepshead minnows (Cyprinodon variegatus). Pp. 109-116 in Bioassay Procedures for the Ocean Disposal Permit Program. U.S. Environmental Protection Agency, Office of Res. and Dev. EPA-600/978-010.

(v) Nimmo, D.E., T.L. Hamaker, and C.A. Sommers. 1978. Entire life-cycle toxicity test using mysids (Mysidopsis bahia) in flowing water. Pp. 64-68 in Bioassay Procedures for the Ocean Disposal Permit Program. U.S. Environmental Protection Agency, Office of Res. and Dev. EPA-600/9-78-010.

(vi) Schimmel, S.C., and D.J. Hansen, 1974. Sheepshead minnow Cyprinodon variegatus: an estuarine fish suitable for chronic (entire life-cycle) bioassays. Proc. 28th Ann. Cong. S.E. Assoc. Game-Fish Comm. Pp. 392-398.

(vii) National Water Quality Laboratory Committee on Aquatic Bioassays. 1971. Recommended bioassay procedure for fathead minnow Pimephales promelas (Rafinesqui) chronic tests. National Water Quality Laboratory, Duluth, Minn. 13 pp. (Revised January 1972.)

(3) Additional information Additional information may be found in the following reference:

(i) Biesinger, K.E. 1974(c). Culturing methods for Daphnia and certain other cladocerans. U.S. Environmental Protection Agency, Environ. Res. Lab., Duluth, Minn.

154A-29 Aquatic ecosystem disruption studies: Tier III.

(a) When required. Data from aquatic ecosystem disruption studies are required by 40 CFR 158.740(d) to support the registration of each end-use formulated product intended for outdoor application and each manufacturing use product that legally may be used to formulate such an end-use product. If, after an analysis of the MPCA's ability to survive and multiply in the environment and what ecological habitat it will occupy, the intended use patterns, and the results of previous nontarget organism and environmental expression tests, it is determined that use of the microbial agent may result in adverse effects on the nontarget organisms in aquatic environments, then testing is required to determine if application of the microbial pest control will be expected to disrupt the balance of populations in the target ecosystem. See 40 CFR 158.50 and 158.740(d) to determine whether these data must be submitted.

(b) Test standards. Specific requirements will be established on a case-by-case basis. Data sufficient to satisfy the general test standards in 150A-3 of this subdivision, and the following test standards:

(1) Test substance. The actual form of the material to be tested is described in Section 154A-2(c)(2) of this document. in addition, any substances used to enhance virulence should be tested along with the test substance.

(2) Test system. The microbial pest control agent will be tested in a complex, realistic microcosm which should be designed in consultation with the Agency. The microcosm should contain members from all trophic levels found in natural aquatic ecosystems.

(2) Test organisms.

(i) Following consultation with the Agency, the registration applicant shall choose a combination of the following species that reflects the species diversity of the target ecosystem:

(A) A typical bottom-feeding fish (e.g., catfish or carp);

(B) A cold-water fish, a warm-water fish, or a marine fish (e.g., brook trout, rainbow trout, bass, bluegill, northern pike, walleye, or sheepshead minnow);

(C) Molluscs (e.g., oyster or freshwater clams);

(D) Crustaceans (e.g., Daphnia spp., shrimp, or crayfish); or

(E) Nymphs (e.g., mayfly).

(c) Reporting and evaluation of data. In addition to the information required by 150A-4 of this subdivision, specific data reporting and evaluation requirements will be established on a case-by-case basis following consultation with the Agency.

(d) Tier progression.

(1) If pathogenic effects are observed then simulated and actual field testing may be required.

(2) If pathogenic effects are observed, additional testing at higher tiers ordinarily is required.

(e) References. The following may contain useful background information for developing acceptable protocols:

(1) Johnson, B.T., and R.A. Schoettger. 1975. A biological model for estimating the uptake, transfer, and degradation of xenobiotics in an aquatic food chain. Fed. Regis. 40 (123):26906-26909. (June 25, 1975.)

(2) Macek, K.J., M.E. Barrows, R.F. Frasny, and B.H. Sleight, III. 1975. Bioconcentration of C^{14}-pesticides by bluegill sunfish during continuous exposure. Pp. 119-142 in Structure-activity correlations in studies of toxicity and bioconcentration with aquatic organisms. G.D. Veith and D.E. Konasevich, eds. Proceedings of a Symposium, Burlington, Ontario, March 11-13, 1975. Sponsored by Standing Committee on Scientific Basis for Water Quality Criteria of the International Joint Commission's Research Advisory Board.

(3) Schimmel, S.C., J.M. Patrick, Jr., and A.J. Wilson. 1977. Acute toxicity to and bioconcentration of endosulfan by estuarine animals. Pp. 241-252 in Aquatic Toxicology and Hazard Evaluation. F.L. Mayer and J.L. Hamelink, eds. STP #634, American Society for Testing and Materials, Philadelphia, Pennsylvania.

154A-30 Special aquatic tests - tissue culture, microorganism/stress interaction tests. [Reserved]

154A-31 Plant studies: Tier III.

(a) When required. Data on the effects of a MPCA on plant growth and development are required by 40 CFR 158.740(d) to support the registration of each end-use formulated product intended for outdoor application and each manufacturing-use product that legally may be used to formulate such an end-use product where the material may transport from the site of application by air, soil, or water. The extent of movement will be determined by the environmental expression tests in Tier II (155A)

(b) Test standards. The test standards shall be developed on consultation with the Agency. These tests will be designed to analyse any significant adverse effects seen in Tier I tests. A range of dose levels will be used in order to determine if the population levels of the MPCA seen in the Tier II studies are able to produce adverse effects. These Tier III protocols may incorporate aspects of Tier IV studies in that the tests may be conducted under simulated or actual environmental conditions.

(c) Reporting. The reporting requirements shall be the same as those in 154A-22(c) of this Subdivision.

(d) Tier progression. If any adverse effects resulting from infection occur or analyses indicate that asymptomatic infection has occurred in Tier III tests, testing at Tier IV (Simulated or Actual Field Testing, 154A-37) is required as specified in 40 CFR 158.740.

154A-32 [Reserved]

TIER IV TESTING

154A-33 Simulated or actual field testing for mammals and birds: Tier IV.

This study is required on a case-by case basis when deleterious effects are observed in Tier III testing. The applicant should consult with the Agency for specific study methodology.

154A-34 Simulated or actual field testing for aquatic organisms: Tier IV.

This study is required on a case-by-case basis when deleterious effects are observed in Tier III testing. The applicant should consult with the Agency for specific study methodology.

154A-35 Simulated or actual field testing for insect predators and parasites: Tier IV

This study is required on a case-by-case basis when deleterious effects are observed in Tier I testing. The applicant should consult with the Agency for specific study methodology.

154A-36 Simulated or actual field testing for insect pollinators: Tier IV

This study will be required on a case-by-case basis when deleterious effects are observed in Tier I testing. The applicant should consult with the Agency for specific study methodology.

154A-37 Simulated or actual field testing for plants: Tier IV

This study will be required on a case-by-case basis when deleterious effects are observed in Tier III testing. The applicant should consult with the Agency for specific study methodology.

Series 155A: Tier II ENVIRONMENTAL EXPRESSION DATA REQUIREMENTS FOR MPCAs

155A-1 General Information

(a) Purpose. Tier II tests are intended to demonstrate whether a MPCA is able to survive or replicate in the environment, thereby indicating which_nontarget_organism(s) may be exposed to the microbial agent, as well as provide an indication of the level of exposure. A determination of the environmental expression of a MPCA includes: an evaluation of the growth of the agent when introduced into a new environment; the way the MPCA may alter its growth habits, take advantage of new environmental conditions or take advantage of changes in the existing equilibrium among microbial species (e.g., in a commensal association, an association in which one species benefits and the other is unaffected). Thus, the expression of a microorganism's presence may result in its insertion into a new habitat and continued propagation in the new habitat.

Terrestrial and aquatic (freshwater and marine) environments are to be considered, as indicated by results from Tier I, the product type and the product usage. Advance consultation with Agency personnel is recommended since requirements may be modified on a case-by-case basis.

The Tier II guidelines consist of descriptions of tests to determine the environmental expression of a MPCA in a terrestrial environment (155A-10), in a fresh water environment (155-11) and in a marine environment (155A-12). If Tier II data is negative (no adverse effects), no further testing is needed.

(b) When Required.

(1) General. Tier II environmental expression data are required by 40CFR 158.740 when toxic or pathogenic effects are observed in maximum hazard testing conducted on nontarget organisms in Tier I tests (154-16 through 24). Table 5 summarizes the relationship between Tier I test results, product type, intended use patterns and the need for Tier II testing. When no adverse effects are observed in Tier I, no Tier II tests are required. Positive data from Tier II provide a basis for further testing as described in Tiers III and IV. Tier II data will be used to establish descriptions of conditions and doses to be used in subsequent testing.

(2) Specific. Specific Tier II data are required when Tier I tests demonstrate adverse effects. See 40 CFR 158.50 and 158.740 to determine whether these data must be submitted.

(i) Terrestrial Environment. Tier II data on the expression of a MPCA in a terrestrial environment are required by 40 CFR 158.740 to support the registration of each end-use formulated product intended for outdoor application on land and each manufacturing-use product that legally may be used to formulate such an end-use product when adverse effects (toxic or pathogenic) are observed in any of the following Tier I tests:

(i) Avian oral pathogenicity/toxicity test: Tier I. (154A-16);

(ii) Avian respiratory pathogenicity test (154A-17);

(iii) Wild mammal toxicity and pathogenicity test (154A-18);

(iv) Plant studies - terrestrial (154A-22);

(v) Testing of toxicity/pathogenicity to insect predators and parasites (154A-23); or

(vi) Honey bee toxicity/pathogenicity test (154A-24).

(2) Freshwater Environment. Tier II data on the expression of a MPCA in a freshwater environment are required by 40 CFR 158.740 to support the registration of each end-use formulated product intended for outdoor application is applied to or may enter freshwater environments and each manfucturing-use product that legally may be used to formulate such an end-use product when adverse effects (toxic or pathogenic) are observed in any of the following Tier I tests:

(i) Avian oral pathogenicity/toxicity test: Tier I. (154A-16);

(ii) Avian respiratory pathogenicity test (154A-17);

(iii) Wild mammal toxicity and pathogenicity test (154A-18);

(iv) Freshwater fish toxicity and pathogenicity testing (154A-19);

(v) Freshwater aquatic invertebrate toxicity and pathogenicity tests (154A-20); or

(vi) Plant studies - aquatic (154A-22).

(3) Marine or Estuarine Environment. Tier II data on the expression of a MPCA in a marine or estuarine environment are required by 40 CFR 158.740 to support the registration of each end-use formulated product intended for outdoor application is applied to or may enter the marine or estuarine environments and each manufacturing-use product that legally may be used to formulate such an end-use product when adverse effects (toxic or pathogenic) are observed in any of the following Tier I tests:

(i) Avian oral pathogenicity/toxicity test: Tier I. (154A-16);

(ii) Avian respiratory pathogenicity test (154A-17);

(iii) Estuarine and marine animal toxicity and pathogenicity test (154A-21); or

(iv) Plant studies - estuarine or marine (154A-22).

(c) Approach. Environmental expression testing consists of simulated terrestrial and aquatic applications of the MPCA. Terrestrial applications are conducted in a contained environment to assess expression in soil and vegetation. Aquatic applications are conducted in aquaria to assess expression in water and sediment. The need for terrestrial, freshwater or marine environmental expression testing under specific conditions depends on:

- The Tier I tests in which adverse effects were observed; and
- The intended use pattern(s) for the MPCA.

Testing is only required to assess environmental expression when susceptible nontarget species may be exposed. Thus, Tier I tests define the need and the environment to be examined. In some cases, no tests may be needed. For example, if adverse effects are noted with terrestrial plants and the intended use pattern calls for use in freshwater only, probably no additional tests would be required. If additional tests are required (e.g., adverse effects with terrestrial insects and a terrestrial use pattern), the timing (e.g., season of use, time of day for release) and location (e.g., climate, soil type) of usage, as well as doses shown to be effective in Tier I, must be considered in selecting Tier II conditions for establishing environmental expression of the test microbe. This simulation of the particular natural environment reflecting the area of use of the product is necessary in order to achieve the overall objective of Tier II testing - determining whether the introduced microorganism will survive, multiply and potentially threaten the nontarget species. The intent is to define potential exposure of susceptible nontarget species to the test product.

Thus, data should be developed which would indicate the dynamics of population fluctuation (i.e. growth and/or survival curves) of the microorganism. The experimental design should consider parameters such as inoculum size, potential for blooms or regrowth, and physical parameters such as relative humidity, pH and temperature.

Table 4 - Summary of Environmental Expression Testing as Determined By Tier I Test Results and Use Patterns

Tier I Test with Positive Results (Test Species)	Proposed Use Patterns for MPCA		
	Terrestrial	Freshwater	Estuarine/Marine
154A-16 and 17 Avian testing - mallard	N/A[1]	F[3]	EM[4]
- quail	T[2]	N/A	N/A
154A-18 Mammalian testing	T	F	N/A
154A-19-21 Fish testing - freshwater spp.	F	F	N/A
- estuarine/marine spp.	EM	EM	EM
154A-20 and 21 Aquatic invertebrate testing - freshwater spp.	F	F	N/A
- estuarine/marine spp.	EM	EM	EM
154A-22 Terrestrial plant testing	T	N/A	N/A
154A-22 Aquatic plant testing - freshwater spp.	F	F	N/A
- estuarine/marine spp.	EM	EM	EM
154A-23 and 24 Terrestrial insect testing	T	N/A	N/A

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[1] N/A: Not applicable - Based on results of Tier I tests and the proposed use pattern, adverse effects are not expected. However, the Agency may require such tests on an individual basis.

[2] T : Tests to determine expression in a terrestrial environment are necessary.

[3] F : Tests to determine expression in a freshwater environment are necessary.

[4] EM : Tests to determine expression in an estuarine or marine environment are necessary.

(a) Applicability. This section outlines the general test standards that apply to the terrestrial, freshwater and aquatic studies. Applicants for registration should also comply with the specific test standards established for the particular test being conducted. In the case of conflict between general and specific test standards, the latter shall govern.

(b) Test standards. Data satisfying the provisions of 155A-2 should meet the general test standards in Subdivision N (160-4 through-6), with the following exception:

MPCA identification and quantification. The most specific available methods for the identification and quantification of the MPCA should be used. The methods used should be consistent with those in 150A-20, Product analysis.

(c) Definitions.

(1) Contained environment. Refers to the use of natural materials from the proposed use-environment (sediment, soil, plants, and marine/ estuarine liquids) arranged as naturally as possible, and held within a plastic, glass, or other container to prevent the escape of the microbial agent.

(2) Substance to be tested. A typical end-use product (EP) or the technical grade of the active ingredient (TGAI) shall be tested.

(3) Observation period. This should be of sufficient duration to unequivocally determine survival, growth or decline (to the limit of detection) of the MPCA in the appropriate environment. Decline of the MPCA should be followed for a sufficient period of time to detect regrowth (see (4) below) of the agent after a prolonged adaptation period.

(4) Regrowth. The number of microorganisms present may decrease initially (perhaps to an undectable level) and then increase (regrowth) to or above the initial cell density. Observations should continue for a period of time sufficient to assure lack of regrowth. In addition, efforts to resuscitate the MPCA (such as enrichment techniques and/or passage in susceptible hosts) should be attempted to support the conclusion that the microbial agent no longer persists in the environment.

155A-2 Reporting and evaluation of data.

(a) Results.

(1) Data collected to determine whether the MPCA is able to survive, persist or replicate in the environment under Tier II test conditions is best expressed in the form of a population growth or decline curve for the MPCA, although other applicable methods of presenting the data may be used.

(2) Test reports should also contain the information designated in the following list, modified as necessary to be applicable to the microbial agent and environment being tested. This information should be given in sufficient detail to adequately define potential impact on growth characteristics of the test organism. In general, the amount of information and kind of data that should be reported in Tier II testing are very similar for any of the environments being examined and are, where applicable, as follows:

(i) type of system and components used;

(ii) source of components;

(iii) equilibrium period before addition of MPCA

(iv) temperature, light requirements, humidity or moisture content, pH of the medium, oxygen requirements;

(v) type of formulation (EP or TGAI);

(vi) application rate or dose level;

(vii) identity of test substance and composition of formulation;

(viii) characteristics of water in aquatic systems;

(ix) method of application;

(x) sampling amount, schedule, and sensitivity of detection;

(xi) tabulation/graphs of population dynamics;

(xii) discussion of test results.

(b) Tier progression. If the results of Tier II expression testing in simulated, contained environments show that the MPCA will survive and persist in the environment under study, thereby exposing non-target organisms to the MPCA, then further testing on the

Tier III level is required. If the results are negative (the microbial agent does not survive or persist), then no further testing is required. Unequivocal determination of negative results may be difficult to prove. Diligent efforts using enrichment techniques may be needed to prove the results are negative and that progression to Tier III is not required.

155A-2 Through 152A-9 [Reserved]

155A-10 Tests to determine expression in a terrestrial environment.

(a) When required. Data on the expression of a microbial pest control agent in a terrestrial environment are required by 40 CFR 158.740 to support the registration of each end-use product intended for outdoor application on land and each manufacturinguse product that legally may be used to formulate such an end-use product when toxic or pathogenic effects are observed in any of the following Tier I tests:

(1) Avian oral pathogenicity/toxicity test: Tier I. (154A-16);

(2) Avian respiratory pathogenicity test (154A-17);

(3) Wild mammal toxicity and pathogenicity test (154A-18);

(4) Plant studies - terrestrial (154A-22);

(5) Testing of toxicity/pathogenicity to insect predators and parasites (154A-23); or

(6) Honey bee toxicity/pathogenicity test (154A-24).

(7) See 40 CFR 158.50 and 158.740 to determine whether these data must be submitted.

(b) Test standards.

(1) Method.

(i) Tests shall be conducted in a greenhouse environment to determine whether the MPCA is able to survive, persist, and replicate in a terrestrial environment consisting of soil and vegetation representative of the proposed use site. The following parameters should be varied to determine their effect on the survival and growth of the MPCA population:

(A) Temperature;

(B) Humidity;

(C) Precipitation (amount, frequency, pH);

(D) Sunlight;

(E) pH (soil and foliar surfaces); and

(F) Nutrients (soil, vegetation).

(ii) The values selected for each parameter listed in paragraph (b)(1)(A) through (F) of this section should be selected to approximate the conditions expected at the intended use site.

(iii) Laboratory studies designed to determine the microbial agent's growth requirements (e.g., temperature, humidity, pH, sunlight, and nutrients) may supplement the greenhouse study described in paragraph (b)(1)(i). Laboratory studies may demonstrate that the MPCA will be unable to survive and the Agency will consider studies on a case-by-case basis to meet the intent of testing in 155-18 in lieu of the greenhouse study.

(2) *Test subscance*. A typical end-use product or the technical grade of the active ingredient shall be tested.

(3) *Test duration*. Data to establish a population decline curve shall be collected at intervals until two half-life determinations have been made or until data establish that the microbial agent population is able to maintain itself in the terrestrial environment at or above the level present immediately after test initiation.

(c) *Reporting and evaluation of data*. The reporting and evaluation provisions are the same as those set forth in 155A-2.

(d) *Tier progression*. If results of this study indicate that the MPCA is able to persist in the terrestrial environment such that the susceptible nontarget organism(s) tested in Tier I are likely to be exposed, then the appropriate testing in Tier III (154A-25, -26, or -31) is required as specified in 40 CFR 158.740.

(e) *References*. The following references contain information for developing acceptable protocols:

(1) Anthony, D.W.; Savage, K.E.; Hazard, E.I.; Avery, S.W.; Boston, M.D.; Oldacre, S.W. (1978) Field test with *Nosema algerae* Vavra and Undeen (Microsporida, Nosematidae) against *Anopheles albimanus* Wiedemann in Panama. Miscel. Publ. Entomol. Soc. Amer. 11:17-28.

(2) Armstrong, J.L.; Knudsen, G.R.; Seidler, R.L. (1987) A microcosm method to assess survival of recombinant bacteria associated with plants and herbivorus insects. Current Microbiol. in press.

(3) Cunningham, J.C. (1970) Persistence of the nuclear polydrosis virus of the eastern hemlock looper on balsam foliage. Insect Pathology Res. Institute. Sault Ste. Marie, Ontario, Canada. Bimonthly Res. Notes 26:24-25.

(4) Elgee, E. (1975) Persistence of a virus of the white-marked tussock moth on balsam fir foliage. Maritime Forest Res. Centre. Fredericton, New Brunswick, Canada. Bimonthly Res. Notes 31:33-34.

(5) Grison, P.; Martouret, D.; Servais, B.; Devriendt, M. (1976) Microbial pesticides and environment. Ann. Zool. Ecol. Anim. 8(2):133-160.

(6) Harcourt, D.J. (1968) Persistence of a granulosis virus of *Pieris rapae* in soil. J. Invert. Path. 11:142-143.

(7) Hukuhara, T.; Namura, H. (1972) Distribution of a nuclear polyhedrosis virus of the fall webworm *Hyphantria cunea* [illegible] soil. J. Invert. Path. 19:308-316.

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155A-11 Tests to determine expression in a freshwater environment.

(a) When required.

(1) Data on the expression of a microbial pest control agent in a freshwater environment are required by 40 CFR 158.740 to support the registration of each end-use product intended for outdoor application on land and each manufacturing-use product that legally may be used to formulate such an end-use product when toxic or pathogenic effects are observed in any of the following Tier I tests:

(i) Freshwater fish toxicity and pathogenicity test (154A-19);

(ii) Freshwater aquatic invertebrate toxicity and pathogenicity test (154A-20); or

(iii) Plant studies - aquatic (154A-22).

(2) Data on the expression of a MPCA in a freshwater environment are required by 40 CFR 158.740 to support the registration of each end-use product intended for outdoor application on fresh water and each manufacturing-use product that legally may be used to formulate such an end-use product when toxic or pathogenic effects are observed in any of the following Tier I tests:

(i) Avian oral pathogenicity/toxicity test: Tier I. (154A-16);

(ii) Avian respiratory pathogenicity test (154A-17);

(iii) Wild mammal toxicity and pathogenicity test (154A-18);

(iv) Freshwater fish toxicity and pathogenicity testing (154A-19);

(v) Freshwater aquatic invertebrate toxicity and pathogenicity tests (154A-20); or

(vi) Plant studies - aquatic (154A-22).

(3) See 40 CFR 158.50 and 158.740 to determine whether these data must be submitted.

(b) Test standards.

(1) Method.

(i) Tests shall be conducted in a simulated aquatic environment (e.g., aquarium with bottom sediment) to determine whether the MPCA is able to survive, persist, and replicate in a freshwater environment consisting of fresh water and bottom sediment representative of the proposed use site. The following parameters should be varied to determine their effect on the survival and growth of the MPCA population:

(A) Temperature;

(B) pH;

(C) Nutrients;

(D) Sunlight;

(E) Oxygen content;

(F) Hardness; and

(G) Turbulence.

(ii) The values selected for each parameter listed in paragraph (b)(1)(A) through (G) of this section should be selected to approximate the conditions expected at the intended use site.

(iii) Specialized laboratory studies designed to determine the MPCA's growth requirements (e.g., temperature, pH, sunlight, and oxygen) may supplement the study described in paragraph (b)(1)(i) of this section. Specialized lab studies may demonstrate that the MPCA will be unable to survive and persist in a freshwater environment. In such instances, the Agency will consider studies on an individual basis to meet the intent of testing in 155A-11 in lieu of the study described in paragraph (b)(1)(i) of this section.

(2) Test substance. A typical end-use product or the technical grade of the active ingredient shall be tested.

(3) Test duration. Data to establish a population decline curve should be collected at intervals until two half-life determinations have been made or until data establish that the microbial agent population is able to maintain itself in a freshwater environment at or above the level present immediately after test initiation.

(c) Reporting and evaluation of data. The reporting and evaluation provisions are the same as those set forth in 155A-2.

(d) *Tier progression*. If results of this study and use patterns information indicate that the MPCA is likely to enter and is able to persist in a freshwater environment such that the susceptible nontarget organism(s) tested in Tier I are likely to be exposed, then the appropriate testing in Tier III (154A-25 through -31) is required as specified in 40 CFR 158.740.

(e) *References*. The following references contain useful information for developing acceptable protocols:

(1) Anonymous. (1975) Impact of the use of microorganisms on the aquatic environment. EPA publication 660-3-75-001. Technical Publications Office, Environmental Protection Agency, National Environmental Res. Center, Corvallis, Oregon 97330.

(2) Anthony, D.W.; Savage, K.E.; Hazard, E.I.; Avery, S.W.; Boston, M.D.; Oldacre, S.W. (1978) Field tests with *Nosema algerae* Vavra and Undeen (Microsporida, Nosematidae) against *Anopheles albinamus* Wiedemann in Panama. Misc. Publ. Entomol. Soc. Amer. 11:17-28.

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155A-12 Tests to determine expression in a marine or estuarine environment.

(a) When required.

(1) Data on the expression of a microbial pest control agent in a marine or estuarine environment are required by 40 CFR 158.740 to support the registration of each end-use product intended for outdoor application on land or in fresh water and each manufacturing-use product that legally may be used to formulate such an end-use product when toxic or pathogenic effects are observed in any of the following Tier I tests:

(i) Estuarine and marine animal toxicity and pathogenicity test (154A-21); or

(ii) Plant studies - estuarine or marine (154A-22).

(2) Data on the expression of a microbial pest control agent in a marine or estuarine environment are required by 40 CFR 158.740 to support the registration of each end-use product intended for outdoor application in marine or estuarine environments and each manufacturing-use product that legally may be used to formulate such an end-use product when toxic or pathogenic effects are observed in any of the following Tier I tests:

(i) Avian oral pathogenicity/toxicity test: Tier I. (154A-16);

(ii) Avian respiratory pathogenicity test (154A-17);

(iii) Estuarine and marine animal toxicity and pathogenicity test (154A-21); or

(iv) Plant studies - estuarine or marine (154A-22).

(3) See 40 CFR 158.50 and 158.740 to determine whether these data must be submitted.

(b) Test standards.

(1) Method.

(i) Tests shall be conducted in a simulated marine or estuarine environment (e.g., aquarium with bottom sediment) to determine whether the MPCA is able to survive, persist, and/or replicate in a marine or estuarine environment consisting of seawater or brackish water and bottom sediment representative of the proposed use site. The following parameters should be varied to determine their effect on the survival and growth of the MPCA population:

(A) Temperature;

(B) pH;

(C) Nutrients;

(D) Salinity;

(E) Sunlight;
(F) Oxygen content; and

(G) Turbulence.

(ii) The values selected for each parameter listed in paragraph (b)(1)(A) through (G) of this section should be selected to approximate the conditions expected at the intended use site.

(iii) Specialized laboratory studies designed to determine the MPCA's growth requirements (e.g., temperature, pH, sunlight, and oxygen) may supplement the study described in paragraph (b)(1)(i) of this section. A specialized lab study(ies) may demonstrate that the MPCA will be unable to survive and persist in a marine or estuarine environment. In such instances, the Agency will consider this study(ies) on an individual basis to fulfill the intent of the testing in 155A-12 in lieu of the study described in paragraph (b)(1)(i) of this section.

(2) Test substance. A typical end-use product or the technical grade of the active ingredient shall be tested.

(3) Test duration. Data to establish a population decline curve should be collected at intervals until two half-life determinations have been made or until data establish that the microbial agent population is able to maintain itself in a marine or estuarine environment at or above the level present immediately after test initiation.

(c) Reporting and evaluation of data. The reporting and evaluation provisions are the same as those set forth in 155A-2. In addition, the following information should be reported:

(1) Any changes in morphology of the microorganism in response to changes in salinity.

(d) Tier progression. If results of this study and use pattern information indicate that the MPCA is likely to enter and is able to persist in a marine or estuarine environment such that the susceptible nontarget organism(s) tested in Tier I is likely to be exposed, then the appropriate testing in Tier III (154A-25 through -31) is required as specified by 40 CFR 158.740.

(e) References. Refer to 154A-11(e).

Series 156A: PRODUCT PERFORMANCE GUIDELINES FOR MICROBIAL PEST CONTROL AGENTS

156A-1 Overview.

Submission of product performance data has been generally waived for most products. It should be noted, however, that the Administrator may require, on a case-by-case basis, product performance data on any specific product whenever he deems that such data are necessary to make proper benefit evaluations for decisions whenever significant risk concerns are identified.

Certain product performance related data on MPCAs have been solicited in these guidelines. These data include: the available information on host spectrum and the time and the minimum effective dosage required for a product to achieve the desired level of pest control or other product performance standard. Such information would ordinarily be developed (and reported) in connection with efficacy studies and is important in the evaluation of nontarget organism safety data.

156A-2 General provisions.

(a) Waiver of data requirements: background and policy. An amendment to FIFRA section 3(c)(5) provides that the Administrator may waive data requirements pertaining to efficacy. This amendment states:

> In considering an application for the registration of a pesticide, the Administrator may waive data requirements pertaining to efficacy, in which event the Administrator may register the pesticide without determining that the pesticide composition is such as to warrant proposed claims of efficacy.

The Agency, in testimony before Congress, stated that it is most concerned about ensuring a product's effectiveness when a lack of efficacy could result in adverse human health effects. In keeping with this concern, the Administrator has deemed that all applications for products not having a direct impact on public health may have their efficacy requirements waived. The Agency is limiting its direct concern to, and requiring efficacy data for, products having health-related use patterns and products proposing new and added uses of chemicals which have been identified as posing a risk of unreasonable adverse effects.

(1) Product performance data will generally only be required by 40 CFR 158.160 for products of the following types:

(i) Uses of antimicrobial agents intended to control pest microorganisms (except bacteria, pathogenic fungi, or viruses living on or in man or other animals) that pose a threat to human health and whose presence cannot readily be observed by the user, including, but not limited to, microorganisms infectious to man in any area of the inanimate environment;

(ii) Uses of fungicides intended for control of organisms that produce mycotoxins.

(iii) Uses of vertebrate control agents intended to control:

(A) Commensal rat and mouse products (Norway, roof and polynesian rat, and house mouse);

(B) Products used to control or disperse birds from buildings, roosts and other areas where they present health hazards;

(C) Products used to control rabies vectors such as bats, skunks, raccoons, and canids; and

(D) Products used to control rodents considered to be plague vectors (ground and tree squirrel, rat, and mouse).

(2) With regard to certain pesticides under suspension or concellation action or subject to a Notice of Special Review under 40 CFR 154.7, the Agency has determined that product performance data are required to be submitted to support any new or additional uses of products that are:

(i) Already under suspension or cancellation action be the Agency for reason that some uses cause human or environmental safety hazards, provided that the risks identified in the suspension or cancellation action also apply to the new uses; or

(ii) Already subject to a Notice of Special Review, provided that the risks identified in the Notice also apply to the new or additional use(s).

(3) For those products for which the Administrator will ordinarily waive the requirement for submittal of efficacy and comparative product performance test data as indicated intends that the registrant will generate and maintain in their records data which is the basis for their label claims.

(i) With regard to conditions where the Agency may request efficacy data but not be limited to these causes for any product, registered or proposed for registration such as when:

(A) A lack of efficacy has been reported for it; or

(B) The Agency needs such data to evaluate benefits of the pesticide (or of alternative pesticides) when substantial risks have been identified; or

(C) Factors exist that make submission of such data necessary or desirable to support the presumption that it is efficacious.

(4) Data on phytotoxicity to the target site, i.e., crops or other desirable plants, are considered part of an efficacy evaluation and submission is thus waived but may be called in on a case by case basis. On the other hand, data on phytotoxicity to crops or other plants that are nontarget sites are considered to be data for hazard evaluation and must be submitted on a case-by case basis as prescribed in 154A-4 and -22. Data on the effects of MPCAs on nontarget plants must be submitted for all such products as described in 154-22.

156A-3 Specific provisions.

(a) The following provisions apply to all microbial pest control agents regardless of whether product performance data submissions are not waived in accordance with 156A-2(a):

(1) The available information on host spectrum shall be reported;

(2) The time required to achieve the desired level of pest control or other product performance standard shall be reported; and

(3) The minimum effective dosage (MED) necessary to achieve the desired level of pest control or other product performance standard shall be reported. The registrant is referred to Subdivision G, Product Performance, for specific guidance and information on data and reporting requirements.

Series 157A: EXPERIMENTAL USE PERMIT GUIDELINES FOR MICROBIAL PEST CONTROL AGENTS

157A-1 Overview.

Data to support applications for Experimental Use Permits (EUPs) for microbial pesticides generally include those data that would ordinarily be generated during the initial stages of product development. For example, most product analysis information would be developed early in the product development stages, and the Tier [illegible] toxicology and nontarget organism toxicity tests would usually be conducted first in preparation for registration. Unless these test results indicate toxic, pathogenic, or other harmful properties, no data on residues or environmental fate would ordinarily be necessary. Product performance data requirements follow the pattern already described in the Subdivision I Guidelines, in which waives data for most products not dealing with public health areas are not required. However, 157A-4(g) also proposes the submittal of data on host spectrum, and time and minimum effective dosage required to achieve the product performance standard.

As indicated in 150A-1(c), "Data requirements for field testing", the Agency recognizes the need to limit testing expenses in the development of microbial pesticides and will make every effort to grant data waiver requests where justified. Anyone planning to submit an EUP should consult with Agency scientists in order to develop an appropriate testing scheme.

In accordance with 40 CFR 172.3, no experimental use permit will be required for non-food-use tests conducted on a cumulative total of not more than 10 acres of land or not more than 1 surface acre of water providing that the water is not used for irrigation, drinking water, or body contact recreational activities, or that the water not contain or affect any fish shellfish or other plants or animals used for food or feed. The Agency is in the process of developing an amendment to 40 CFR 172 to require notification prior to conducting small scale field tests of certain genetically altered MPCAs. In the meantime, the Agency is following the policy published in the June 26, 1986 FEDERAL REGISTER (51 FR 23302). Under this policy, a written notification is to be submitted to the Agency prior to conducting small scale field tests of genetically altered or nonindigenous MPCAs. The Agency has 90 days to determine if an EUP application is required for this field test.

157A-2 Scope and intent.

This section series deals with the data necessary to support the application for an Experimental Use Permit for microbial pest control agents. These guidelines are based on FIFRA section 5 and

40 CFR Part 172, and they closely match the guidelines in Subdivision I in many respects. For further information on scope and intent, refer to 110-1 of Subdivision I.

157-3 General Provisions.

In developing plans and information for an Experimental Use Permit application, the applicant should carefully review section series 110 and 111 of Subdivision I. With the exception of several cross-references to specific data in other subdivisions of the guidelines, the provisions of those sections of Subdivision I apply to MPCAs as well as to conventional pesticides.

When requesting preliminary assistance from Agency scientists in determining a data testing scheme, as much of the following information on the MPCA as possible should be available. This kind of information will be used to determine the specific tests needed or to determine the appropriateness of approving test waiver requests.

(1) The identity of the MPCA including:
(i) Characteristics.
(ii) Means and limit of detection.
(2) Description of its natural habitat including information on:
(i) Predators.
(ii) Parasites.
(iii) Competitors.
(3) Information on the host range, with an assessment of infectivity and pathogenicity to nontarget organisms.
(4) Information on the population dynamics of the microorganism in the environment.
(5) A description of the proposed testing program including:
(i) The purpose or objectives of the proposed testing.
(ii) Designation of the pest organism(s) involved.
(iii) The State(s) in which the proposed program will be conducted.
(iv) The specific identity of the exact location of the test sites(s) (including proximity to residences and human activites, surface water, etc.)
(v) The crops, fauna, flora, geographical description of sites, modes, dosage rates, frequency, and situation of application on or in which the pesticide is to be used.
(vi) The amount of pesticide product proposed for use.
(vii) The method of application.
(viii) A comparison of the natural habitat of the microorganism with the proposed test site.
(ix) The number of acres, structural sites, or animals/plants by State, to be treated or included in the area of experimental use.
(x) Procedures to be used to protect the test area from intrusion by unauthorized individuals.
(xi) The proposed dates or period(s) during which the testing program is to be conducted, and the manner in which superviaion of

the program will be accomplished.

(xii) Description of procedures for monitoring the microorganism within and adjacent to the test site during the test.

(xiii) The method of disposal or sanitation of plants, animals, soils, etc., that were exposed during and after the field test.

(xiv) Means of evaluating potential adverse effects and methods of controlling the microorganism if detected beyond the test area.

(6) A statement of composition for the formulation to be tested, giving:

(i) The name and percentage by weight of each ingredient, active and inert.

(ii) Production methods.

(iii) Extraneous microorganisms present as contaminants.

(iv) Amount and potency of any toxin present.

(v) The number of viable microorganisms per unit weight or volume of the product (or other appropriate system for designating the quantity of active ingredient).

The following information applies to genetically altered MPCAs:

(7) Description of the methods used to genetically alter the microorganism.

(8) The identity and location of the rearranged or inserted/deleted gene segment(s) in question (host source, nature, base sequence data or restriction enzyme map of the gene(s).

(9) Information on the control region of the gene(s), and a description of the new trait(s) or characteristic(s) that are expressed.

(10) Data on the potential for genetic transfer and exchange with other organisms and on genetic stability of any inserted sequence.

(11) Data on the relative environmental competitiveness compared to the parental strains.

157A-4 Specific data requirements.

(a) General.

(1) The following types of data are required by 40 CFR 158.740 to support an application for an Experimental Use Permit for a MPCA:

(i) Product analysis; refer to paragraph (b) of this section;

(ii) Residues; refer to paragraph (c) of this section;

(iii) Toxicology; refer to paragraph (d) of this section;

(iv) Nontarget organisms; refer to paragraph (e) of this section;

(v) Environmental fate; refer to paragraph (f) of this section; and

(vi) Product performance; refer to paragraph (g) of this section.

(2) General policies related to data necessary to support an Experimental Use Permit are delineated in Subdivision I, 112-1.

(b) Product analysis data. To support an application for an Experimental Use Permit, the data outlined in 151A-20 through -26 apply to microbial pest control agents.

(c) Residue data. For microbial pest control agents used on food or feed crop or whose use is expected to result in residues in or on food or feed, no data are required unless Tier I toxicology studies conducted under section 152A of this subdivision indicate a potential for human hazard. Residue data developed in accordance with section 153A of this subdivision would then be required to obtain a temporary tolerance.

(d) Toxicology data. The following data are required by 40 CFR 158.740 to support an application for an Experimental Use Permit:

(1) For all MPCAs:

(i) Acute oral toxicity/pathogenicity (152A-10);

(ii) Acute dermal toxicity/pathogenicity (152A-11);

(iii) Acute pulmonary toxicity/pathogenicity (152A-12);

(iv) Acute intravenous, toxicity/pathogenicity (152A-13); and

(v) Primary eye irritation (152A-14).

(vi) Hypersensitivity incidences (152A-15).

(2) For viruses used as MPCAs on food crops:

(i) All studies listed in paragraph (d)(3) of this section;

(ii) Tissue culture (152A-16).

(e) Nontarget organism data. To support an application for an Experimental Use Permit, nontarget organism data developed in Tier I studies of microbial pest control agents as described in section series 154A are required as specified in 40 CFR 158.740.

(f) Environmental fate and expression data. To support an application for an Experimental Use Permit, data from environmental fate and expression studies according to section series 155A are required by 40 CFR 158.740 for those microbial pest control agents whose Tier I nontarget organism test results (from section series 154A) indicate that Tier II studies for environmental fate and expression should be conducted. For those pest control agents whose Tier I nontarget organism test results indicate no Tier II studies are necessary, no environmental fate and expression data are required for the application of a Permit. In those instances where field data from Tier II studies are required in section series 155A for a Permit, field data from contained simulated environments or field data conducted on similar microorganisms may suffice in lieu of extensive field data; this policy is needed to preclude development of extensive field data without a permit in order to obtain information necessary to get a Permit.

(g) Product performance data.

(1) General. In general, product performance data will not be required by 40 CFR 158.740 to support the issuance of an Experimental Use Permit.

(2) Exceptions.

(i) Initial permits. Efficacy data may be required, on a case-by-case, for the following use categories:

(A) Public health uses dealing with pest microorganisms, and vertebrate control agents; and

(B) Use of cancelled or suspended pesticides.

(ii) Extensions, renewals, and amendments. Summaries of product performance data collected under an Experimental Use Permit may be requested on a case-by-case basis by the Agency for purposes of making:

(A) Determinations as to the need for additional quantities of product requested by the applicant;

(B) Evaluations of requests for Permit extensions; and

(C) Assessments of requests for Permit renewals.

Series 158A: LABEL DEVELOPMENT

158A-1 Product label requirements.

Microbial pest control agents are generally subject to all applicable labeling provisions described in Subdivision H - Labeling Requirements for Pesticides and Devices. Labeling for microbial agents differs principally with respect to the ingredient statement. Some instruction regarding ingredient statements for MPCAs can be derived from 151A-1 through -17 (Product Analysis) of this subdivision. Also, see 156A-3 regarding label claims, directions, precautions, and restrictions in relation to use pattern

APPENDIX Microbial Pesticides Data Requirements

§ 158.170 Microbial pesticides - Product analysis data requirements.

(1) Microbial pesticides product analysis data requirements

		GENERAL USE PATTERNS									TEST SUBSTANCE		
Kind of Data Required	Notes	Terrestrial Food Crop	Terrestrial Non Food	Aquatic Food Crop	Aquatic Non-Food	Greenhouse Food Crop	Greenhouse Non Food	Forestry	Domestic Outdoor	Indoor	Data to Support MP	Data to Support EP	Guidelines Reference Numbers
Product Identity		[R]	[R]	[R]	[R]	[R]	[R]	[R]	[R]	[R]	MP	EP*	151A-10
Manufacturing Process	(i)	[R]	[R]	[R]	[R]	[R]	[R]	[R]	[R]	[R]	MP & TGAI	EP* & TGAI	151A-11
Discussion of Formation of Unintentional Ingredients	(ii)	[R]	[R]	[R]	[R]	[R]	[R]	[R]	[R]	[R]	MP & TGAI	EP* & TGAI	151A-12
Analysis of Samples	(iii)	[CR]	[CR]	[CR]	[CR]	[CR]	[CR]	[CR]	[CR]	[CR]	MP & TGAI	EP* & TGAI	151A-13
Certification of Limits		[R]	R	[R]	R	[R]	R	R	R	R	MP	EP*	151A-15
Physical and Chemical Properties		[R]	[R]	[R]	[R]	[R]	[R]	[R]	[R]	[R]	MP & TGAI	EP* & TGAI	151A-16
Submittal of Samples	(iv)	[CR]	[CR]	[CR]	[CR]	[CR]	[CR]	[CR]	[CR]	[CR]	MP & TGAI PAI	EP*, TGAI & PAI	151A-17

KEY: R = Required; CR = Conditionally Required; MP = Manufacturing-use product; EP* = End-use product, (asterisk identifies those data requirements that end-use applicants (i.e., "formulators") must satisfy, provided that their active ingredient(s) is (are) purchased from a registered source); TGAI = Technical Grade of the Active Ingredient; [] = Brackets (i.e., [R], [CR] indicate data requirements that apply when an experimental use permit is being sought.

(i) If an experimental use permit is being sought, a schematic diagram and/or description of the manufacturing process will suffice if the pesticide is not already under full scale production.

(ii) If the product is not already under full scale production and an experimental use permit is being sought, a discussion of unintentional ingredients shall be submitted to the extent this information is available.

(iii) Required to support registration of each manufacturing-use product and end use products produced by an integrated formulation system. Data on other end use products will be required on a case-by-case basis. For pesticide in the production stage, a rudimentary product analytical method and data will suffice to support an experimental use permit.

(iv) Routinely required for products produced by an integrated formulation system. Required on a case-by-case basis for other products or materials.

(2) Microbial pesticides residue data requirements

		GENERAL USE PATTERNS									TEST SUBSTANCE		
Kind of Data Required	Notes	Terrestrial Food Crop	Terrestrial Non-Food	Aquatic Food Crop	Aquatic Non-Food	Greenhouse Food Crop	Greenhouse Non-Food	Forestry	Domestic Outdoor	Indoor	Data to Support MP	Data to Support EP	Guidelines Reference Number
Residue Data	(i)	[CR]	[CR]	[CR]	[CR]	[CR]	[CR]	[CR]	[CR]	[CR]	-	-	153A

Key: CR = Conditionally Required Data; End-use product; MP = Manufacturing-use product; [] = Brackets (i.e., [CR]) indicate data requirements that apply when an experimental use permit is being sought.

(i) Residue data requirements shall apply to microbial pesticides when Tier II or Tier III toxicology data are required.

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(3) Microbial pesticides toxicology data requirements.

		GENERAL USE PATTERNS									TEST SUBSTANCE	
Kind of Data Required	Notes	Terrestrial Food Crop	Terrestrial Non Food	Aquatic Food Crop	Aquatic Non-Food	Greenhouse Food Crop	Greenhouse Non-Food	Forestry	Domestic Outdoor	Indoor Use	Data to Support MP and EP	Guideline Reference Numbers
Tier I												
Acute oral tox/path		[R]	[R]	[R]	[R]	[R]	[R]	[R]	[R]	[R]	TGAI	152A-10
Acute dermal tox		R	R	R	R	R	R	R	R	R	MP & EP	152A-11
Acute pulmonary tox/path		[R]	[R]	[R]	[R]	[R]	[R]	[R]	[R]	[R]	TGAI	152A-12
Acute intravenous tox/path	(i)	[R]	[R]	[R]	[R]	[R]	[R]	[R]	[R]	[R]	TGAI	152A-13
Primary eye irrit.	(ii)	[R]	[R]	[R]	[R]	[R]	[R]	[R]	[R]	[R]	MP & EP	152A-14
Hypersensitivity incidents	(iii)	R	R	R	R	R	R	R	R	R	-	152A-15
Cell culture	(iv)	[R]	[R]	[R]	[R]	[R]	[R]	[R]	[R]	[R]	MIF	152A-16
Tier II												
Acute tox	(v)	CR	CR	CR	CR	CR	CR	CR	CR	CR	-	152A-20
Subchronic tox/path	(vi)(vii)	CR	CR	CR	CR	CR	CR	CR	CR	CR	-	152A-21
Tier III												
Reproductive/fertility effects	(viii)	CR	CR	CR	CR	CR	CR	CR	CR	CR	-	152A-30
Oncogenicity	(ix)	CR	CR	CR	CR	CR	CR	CR	CR	CR	-	152A-31
Immunodeficiency	(x)	CR	CR	CR	CR	CR	CR	CR	CR	CR	-	152A-32
Primate infectivity/path	(xi)	CR	CR	CR	CR	CR	CR	CR	CR	CR	-	152A-33

KEY: R = Required; CR = Conditionally Required; MIF = Most Infective Form; MP = Manufacturing-use Product; EP = End-use Product; TGAI = Technical Grade of the Active Ingredient; [] = Brackets (i.e., [R], [CR]) indicate data requirements that apply when an experimental use permit is being sought.

(a) If an MP is different than the TGAI, then an acute oral toxicity study (81-1) and an acute inhalation study (81-3) as required in 158.340 for chemical pesticides is to be done with the MP as the test substance. If the EP is different than the MP and the TGAI, then an acute oral toxicity study (81-1) and an acute inhalation study (81-3) as required in 158.340 for chemical pesticides is to be done with the EP as the test substance. End-use applicants (i.e., "formulators") must satisfy these data requirements for the end-use products provided that their active ingredient(s) is (are) purchased from a registered source.

(b) Notes.

(i) Data not required for products whose active ingredient is a virus.

(ii) Data not required if it is demonstrated (usually by product label statement inclusion) that workers/applicators eyes will be appropriately protected during times of exposure to aerosols of the MPCA.

(iii) Hypersensitivity incidents must be reported, if they occur.

(iv) Data required for products whose active ingredient is a virus.

(v) Data required when toxicity, in the absence of pathogenicity and significant infectivity is observed in Acute oral, dermal, and pulmonary studies (Tier I). Route(s) of exposure correspond to routes where toxicity was observed in Tier I studies.

(vi) Data required when significant infectivity and/or unusual persistence is observed in the absence of pathogenicity or toxicity in Tier I studies. Routes of exposure (oral and/or pulmonary) correspond to routes in Tier I studies.

(vii) Also may be required to evaluate adverse effects due to microbial contaminants or to toxic by-products.

(viii) Data required when any of the following criteria are met:

1) Significant infectivity of the MPCA was observed in test animals in the Tier II subchronic study (152A-22) and in which no significant signs of toxicity or pathogenicity were observed.

2) The MPCA is a virus which can persist or replicate in mammalian cell culture lines (152A-19).

3) The MPCA is not amenable to thorough taxonomic classification, and is related to organisms known to be be parasitic for mammalian cells.

4) The MPCA preparation is not sufficiently well purified, but it is indicated that the preparations may containe contaminants which are parasitic for mammals.

(ix) Data may be required for products known to contain, or suspected to contain, oncogenic viruses.

(x) Data may be required for products known to contain or suspected to contain, viruses that can interact in an adverse manner with components of mammalian immune systems.

(xi) Data may be required for products that are known to contain, or are suspected of containing intracellular parasites of mammalian cells, or for products that exhibit pathogenic characteristics in Tier 1 or for known human pathogens that have been "disarmed".

(4) Microbial pesticides non-target organism and environmental expression data requirements.

		GENERAL USE PATTERNS									TEST SUBSTANCE		
Kind of Data Required	Notes	Terrestrial		Aquatic		Greenhouse		Forestry	Domestic Outdoor	Indoor Use	Data to Support MP	Data to Support EP	Guidelines Reference Numbers
		Food Crop	Non Food	Food Crop	Non-Food	Food Crop	Non Food						
Tier I													
Avian oral	(i,ii,iii)	[R]	[R]	[R]	[R]	CR	CR	[R]	[R]	CR	TGAI	TGAI	154A-16
Avian inhalation test	(iv)	CR	CR	CR	CR	CR	CR	CR	CR	CR	TGAI	TGAI	154A-17
Wild mammal testing	(v)	CR	CR	CR	CR	-	-	CR	CR	-	TGAI	TGAI	154A-18
Freshwater fish testing	(i,ii)	[R]	[R]	[R]	[R]	CR	CR	[R]	CR	CR	TGAI	TGAI	154A-19
Freshwater aquatic invertebrate testing	(i)	[R]	[R]	[R]	[R]	CR	CR	[R]	CR	CR	TGAI	TGAI	154A-20
Estuarine and marine animal testing	(vi)	CR	CR	CR	CR	-	-	CR	CR	-	TGAI	TGAI	154A-21
Nontarget plant studies		[R]	[R]	[R]	[R]	-	-	[R]	[R]	CR	TGAI	TGAI	154A-22
Nontarget insect testing		[R]	[R]	[R]	[R]	CR	CR	[R]	[R]	-	TGAI	TGAI	154A-23
Honey bee testing		[R]	[R]	[R]	[R]	CR	CR	[R]	[R]	-	TGAI	TGAI	154A-24
Tier II													
Terrestrial environmental testing	(vii)	CR	CR	CR	CR	-	-	CR	CR	-	TGAI or TEP	TGAI or TEP	155A-18
Freshwater environmental expression tests	(viii)	CR	CR	CR	CR	-	-	CR	CR	-	TGAI or TEP	TGAI or TEP	155A-19
Marine or estuarine environmental expression tests	(ix,x)	CR	CR	CR	CR	-	-	CR	CR	-	TGAI or TEP	TGAI or TEP	155A-20
Tier III													
Terrestrial Wildlife & Aquatic Organism Testing	(xi)	CR	CR	CR	CR	-	-	CR	CR	-	TGAI or TEP	TGAI or TEP	154A-25
Avian Chronic pathogenicity and reproduction test	(xii)	CR	CR	CR	CR	-	-	CR	CR	-	TGAI	TGAI	154A-26
Aquatic invertebrate range testing	(xiii)	CR	CR	CR	CR	-	-	CR	CR	-	TGAI	TGAI	154A-27
Fish Life Cycle Studies	(xiii)	CR	CR	CR	CR	-	-	CR	CR	-	TGAI	TGAI	154A-28
Aquatic Ecosystem Test	(xv)	CR	CR	CR	CR	-	-	CR	CR	-	TGAI	TGAI	154A-29
Special Aquatic Tests (Reserved)		-	-	-	-	-	-	-	-	-	-	-	154A-30
Nontarget Plant Studies	(xvi)	CR	CR	CR	CR	-	-	CR	CR	-	TGAI	TEP	154A-31
Tier IV	(xvii)												
Simulated and Actual Field Tests (Birds, (Mammals)	(xiv)	CR	CR	CR	CR	-	-	CR	CR	-	TEP	TEP	154A-33
Simulated and Actual Field Tests (Aquatic Organisms)	(xviii, xix)	CR	CR	CR	CR	-	-	CR	CR	-	TEP	TEP	154A-34
Simulated and Actual Field Tests (Insect Predators, Parasites)	(xviii, xix)	CR	CR	CR	CR			CR	CR	-	TEP	TEP	154A-35
Simulated and Actual Field Tests (Insect Pollinators)	(xviii, xix)	CR	CR	CR	CR			CR	CR	-	TEP	TEP	154A-36

KEY: R = Required
CR = Conditionally Required
[] = Brackets (i.e., [R], [CR]) indicates data requirements that apply to products for which an experimental use permit is being sought.
MP = Manufacturing-use Product
TEP = Typical End-Use product
TGAI = Technical grade of the active ingredient.
EP = End-use product, PAI = "Pure" active ingredient.

Notes.

(i) Tests for pesticides intended solely for indoor application will be required on a case-by-case basis, depending on use pattern, production volume, and other pertinent factors.

(ii) Preferable test species are: bobwhite quail or mallard for avian acute oral and inhalation studies; rainbow trout for freshwater fish studies.

(iii) An injection route of exposure may be used in lieu of the oral route in some cases for the avian acute study.

(iv) Data required when the nature of the MPCA and/or its toxins indicates potential pathogenicity to birds.

(v) Required on a case-by-case basis if results of tests required by paragraph (c)(1) of this section are inadequate or inappropriate for assessment of hazards to wild animals.

(vi) Required when product is intended for direct application into the estuarine or marine environment or expected to enter this environment in significant concentrations because of expected use or mobility pattern.

(vii) Required when toxic or pathogenic effects are observed in any of the following Tier I tests for microbial pest control agents:

(A) Avian acute dose oral or avian inhalation tests.
(B) Wild mammals toxicity and pathogenicity test.
(C) Plant studies - terrestrial.
(D) Honey bee toxicity/pathogenicity test.
(E) Testing for toxicity/pathogenicity to insect predators and parasites.

(viii) Required when toxic or pathogenic effects are observed in any of the following Tier I test for microbial pest control agents:

(A) Freshwater fish toxicity and pathogenicity testing.
(B) Freshwater aquatic invertebrate toxicity and pathogenicity test.
(C) Plant studies - aquatic.

(ix) Required if product is applied on land or in fresh water and toxic or pathogenic effects are observed in any of the following Tier I tests for microbial pest control agents:

(A) Estuarine and marine animal toxicity and pathogenicity test.
(B) Plant studies - estuarine or marine.

(x) Required if product is applied in marine or estuarine environments and toxic or pathogenic effects are observed in any of the following Tier I tests:

(A) Avian acute oral test.
(B) Avian inhalation test.
(C) Estuarine and marine animal toxicity and pathogenicity test.

(xi) Required when toxic effects on nontarget terrestrial wildlife or aquatic organisms are reported in one or more Tier I tests and results of Tier II tests indicate exposure of the microbial agent to the affected nontarget terrestial wildlife or aquatic organisms.

(xii) Required when:

(A) Pathogenic effects are observed in Tier 1 avian tests.
(B) Chronic, carcinogenic, or teratogenic effects are reported in tests required in (c)(1) of this section for evaluating hazard to humans and domestic animals.
(C) Tier II Environmental expression testing indicates that exposure of terrestrial animals to the microbial agent is likely.

(xiii) Required when product is intended for use in water or expected to be transported to water from the intended use site, and when pathogenicity or infectivity was observed in Tier I aquatic tests.

(xiv) Required when both of the following conditions are met:

(A) Pathogenic effects at actual or expected field residue exposure levels are reported in Tier III.
(B) The Agency determines that quarantine methods will prevent the microbial pest control agent from contaminating areas adjacent to the test area.

(xv) Required if, after an analysis of the microbial agent's properties, the individual use patterns, and the results of previous nontarget organism and environmental expression tests, it is determined that use of the microbial agent may result in adverse effects on the nontarget organisms in aquatic environments, including those of the water column and bottom sediments. When a microbial pest control agent is used in or is expected to transport to water from the intended use site, major considerations for requiring these infectivity tests include, but are not limited to:

(A) Infectivity or pathogenicity demonstrated in previous testing.
(B) Viability of the microorganism in natural waters as demonstrated in Tier II tests.

(xvi) Required if the product is transported from the site of application by air, soil, or water or transmission by other animals, and nontarget plant pathogenicity is observed in Tier I tests. The exent of movement will be determined by the environmental expression tests in Tier II.

(xvii) The Agency expects that Tier IV requirements would be imposed retrospectively - after product registration as post registration monitoring, since it is unlikely a registrant would pursue registration of a microbial agent posing potential hazards such that testing beyond Tier III is required.

(xviii) Short term simulated or actual field studies are required when it is determined that the product is likely to cause adverse short-term or acute effects, based on consideration of available laboratory data, use patterns, and exposure rates.

(xix) Data from a long-term simulated field test (e.g., where reproduction and growth of confined populations are observed) and/or an actual field test (e.g., where reproduction and growth of natural populations are observed) are required if laboratory data indicate adverse long-term, cumulative, or life-cycle effects may result from intended use.